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PRESERVED NEURAL FUNCTIONS IN POST-ANOXIC COMA AND THEIR ADDED VALUE FOR THE PREDICTION OF COGNITIVE AND FUNCTIONAL OUTCOME

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PRESERVED NEURAL FUNCTIONS IN POST-ANOXIC COMA AND THEIR ADDED VALUE FOR THE PREDICTION OF COGNITIVE AND FUNCTIONAL OUTCOME

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Lorenz Hirt

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ABSTRACT

Post-anoxic coma after cardiac arrest poses critical challenges to clinicians and families regarding the severity of cerebral insult and the potential of recovery. In such an altered state of consciousness, electroencephalography (EEG), assessing spontaneous and evoked cerebral activity without patient's active participation, provides crucial information about the brain condition. In particular, previous studies showed preserved neural responses in simple and more complex paradigms involving mainly auditory stimuli in some post-anoxic comatose patients, and in particular in those who will survive. These results suggest that the investigation of brain functions during coma can be used to predict patient's prognosis and that consciousness may be not required for low-level processing of sensory stimuli.

However, to which extent the unconscious brain can process complex stimuli, and how brain responses during coma are associated with other clinical markers of cerebral condition and with detailed functional outcome of the survivors remains under-investigated. In the present thesis, I address these points through three experimental studies.

In the first one, I assessed the clinical evolution of patients showing an initial non-reactive EEG, defined as the absence of any visible change in the EEG signal in response to strong nociceptive or auditory stimulation. Despite an extremely poor outcome, patients recovering EEG reactivity on the second day of coma were more likely than the others to also regain other brain functions, suggesting at least partial clinical recovery in these patients.

In the second study, I investigated whether the progression of cerebral responses to auditory stimuli during coma could be predictive of the functional recovery of the survivors. My results indicate that patients improving auditory discrimination display better cognitive performances and better long-term outcome as compared with the others. In addition, the extent of the progression was directly associated with the extent of the recovery, suggesting a close relation between brain's condition during early coma and subsequent functional recovery.

Finally, in the third study, I challenged current opinions regarding the impossibility of learning in the absence of consciousness, by showing that some deeply comatose patients exhibited neural signs of expectancy of a specific stimulus. These patients presented a reactivation of the EEG activity elicited by a sound, precisely when the sound was expected after a tone, indicating the creation of an association between the tone and the following sound.

In summary, my results suggest that the comatose brain can process a large variety of stimuli, shedding new light into the cerebral functions in this clinical state, and that its signal may provide reliable and detailed prognostic information.

RÉSUMÉ

Le coma après arrêt cardio-respiratoire soulève des questions essentielles pour les proches et l'équipe médicale concernant la sévérité de l'atteinte cérébrale et le potentiel de récupération. Dans ce contexte, l'électroencéphalographie (EEG), évaluant l'activité cérébrale spontanée et évoquée sans la participation du patient, fournit des informations cruciales concernant l'état du cerveau. Certains patients comateux, et en particulier ceux qui survivent, montrent une activité neuronale préservée en réponse à des paradigmes simples et plus complexes impliquant des stimuli principalement auditifs. L'investigation des fonctions cérébrales durant le coma peut donc être utile pour prédire le pronostic et la conscience ne semble pas nécessaire pour le traitement de certaines tâches simples.

Cependant, il est encore inconnu à quel point le cerveau inconscient peut traiter des stimuli complexes, et comment les réponses cérébrales pendant le coma sont associées avec d'autres marqueurs cliniques de l'état cérébral et avec le devenir fonctionnel (outcome) des survivants. Ce sont ces questions que je vais traiter à travers trois études expérimentales.

Dans la première, j'ai examiné l'évolution clinique des patients démontrant initialement un EEG non-réactif, définit comme l'absence de changement visible dans le signal EEG en réponse à de fortes stimulations nociceptives ou auditives. En dépit d'un outcome extrêmement sombre, les patients qui récupèrent la réactivité EEG au deuxième jour du coma ont plus de chance que les autres de récupérer également d'autres fonctions cérébrales, suggérant un rétablissement clinique au moins partiel chez ces patients.

Dans la deuxième étude, j'ai investigué si la progression des réponses cérébrales à des stimuli auditifs pendant le coma pouvait prédire la récupération fonctionnelle des survivants. Mes résultats indiquent que les patients qui améliorent la discrimination auditive montrent de meilleures performances cognitives et un meilleur outcome à long-terme par rapport aux autres. De plus, l'étendue de la progression est directement associée à l'étendue de la récupération fonctionnelle, suggérant une forte relation entre l'état du cerveau dans le coma précoce et l'outcome.

Finalement, au cours de la troisième étude, j'ai remis en question l'opinion selon laquelle il serait impossible d'apprendre de nouvelles informations en l'absence de conscience. J'ai montré que certains patients, alors qu'ils étaient profondément comateux, présentaient une réactivation de l'activité EEG en réponse à un son, précisément au moment où le son était attendu après un bip, indiquant la création d'une association entre le bip et le son suivant.

En résumé, mes résultats suggèrent que le cerveau comateux peux traiter une large variété de stimuli, amenant de nouvelles perspectives de cet état clinique, et que son signal peut fournir des informations fiables et détaillées pour le pronostic.

LIST OF ABBREVIATIONS

AEP	auditory evoked potential
DOC	disorders of consciousness
EEG	electroencephalography
ERP	event related potential
FOUR	full outline of unresponsiveness
GCS	Glasgow coma scale
MMN	mismatch negativity
NSE	neuron-specific enolase
SSEP	somato-sensory evoked potentials
TH	therapeutic hypothermia

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1. INTRODUCTION

1.1. Post-anoxic coma

Coma is a state of unconsciousness and unresponsiveness to the environment characterized by the absence of arousal (Laureys *et al.*, 2004). In coma, patients lie with eyes closed, cannot be aroused even in response to vigorous stimulation and have no awareness of self or surroundings (Posner *et al.*, 2007). The two main mechanisms of coma are bihemispheric diffuse cortical or white matter damage after neuronal or axonal injury, or brainstem lesions affecting the subcortical reticular arousing system involving several nuclei (ponto-mesencephalic tegmentum relaying through the thalami) (Laureys *et al.*, 2009). In particular, grey-matter metabolism is drastically decreased as compared to the normal range (Laureys *et al.*, 2004). Etiologies include diffuse and metabolic brain dysfunction, such as in encephalitis or anoxia, or focalized cortical or subcortical lesions after strokes or tumors (Posner *et al.*, 2007). An individual is declared in coma when the unconscious condition persists for at least 1 hour, differently from transient states of unconsciousness due to syncope or concussion (Laureys *et al.*, 2009). Survivors may awake within 2-4 weeks after coma onset, depending on coma etiology. The gradual recovery of consciousness can be slow and sometimes it does not progress beyond vegetative state or minimally conscious state (Laureys *et al.*, 2004). Persistent vegetative state (or unresponsive wakefulness syndrome) differs from coma in that patients are awake, with eyes open, but without any awareness of themselves or the environment, for at least one month after the insult (Laureys *et al.*, 2010). Minimally conscious state indicates awake patients showing reproducible but limited evidence of awareness of the self or the environment, as for example being able of following simple commands or exhibiting purposeful behavior (Giacino *et al.*, 2002; Laureys *et al.*, 2004, 2009).

Coma after cardiac arrest is the first cause of coma in the intensive care unit (Oddo, 2015) and is referred to as post-anoxic coma or anoxic-ischemic encephalopathy. Post-anoxic coma usually lasts for 1 to 15 days and occurs in 80% of successfully resuscitated patients (Madl and Holzer, 2004). Cardiac arrest is a severe condition concerning 8'000 to 10'000 people each year in Switzerland, with a survival rate of less than 10% (Katz *et al.*, 2005). If cardiac arrest is witnessed and an external cardiopulmonary resuscitation is performed, around 17 to 49% of patients will recover a spontaneous cardiac rhythm and be conducted to a hospital intensive care station (Madl and Holzer, 2004). Despite clinical advances in therapeutic interventions and increasing survival rates (Fugate *et al.*, 2012), around 50% of these patients do not survive until hospital discharge, the main cause of death being brain injury (Dragancea *et al.*, 2013). In the following sections, I will refer to death as poor outcome and survival as good outcome, unless specified differently.

1.1.1. Pathophysiology of brain injury

The generalized reduction of blood flow (i.e. global ischemia) following cardiac arrest affects all organs, and in particular the brain, which is one of the most sensitive to ischemia. In global brain ischemia, the drastic diminution of cerebral blood flow over the entire brain is insufficient to meet the metabolic demands of cerebral tissue in oxygen and glucose supply, having detrimental effects for the function and integrity of the brain. A complete deprivation of oxygen supply is referred to as anoxia and occurs quickly after a sustained cardiac arrest, triggering a cascade of chemical events ultimately leading to neuronal death.

The pathophysiology of global brain ischemia is complex and multifactorial, and the extent of cerebral lesions is determined by two distinct phases of injury. The first phase, called "primary ischemic injury", is caused by the initial acute lack of blood (ischemia) and oxygen (anoxia), leading to immediate cell death. Considering that the brain has no energy stores and that brain tissues are unable to sustain anaerobic metabolism, the lack of oxygen and glucose has immediate severe consequences on brain cells (Hofmeijer and Van Putten, 2012). Within the first minutes after cardiac arrest, a complex cascade of events, including the stop of mitochondrial oxidative phosphorylation, depletion of adenosine-triphosphate and accumulation of lactate, leads to a breakdown of the blood-brain barrier, thus altering cellular and organ osmolality and resulting in the formation of brain oedema (Chalkias and Xanthos, 2012).

The second phase, referred to as "second reperfusion injury", is the consequence of the pathophysiological processes initiated in the primary injury and set off by blood flow reperfusion (Engelhard and Werner, 2011). This may damage previously viable ischemic tissues by forming toxic reactive oxygen species in reaction to the accumulation of hypoxanthine during ischemia (Collard and Gelman, 2001). Actually, most of the brain damage caused by ischemia are believed to occur during secondary reperfusion stage (White *et al.*, 2000).

Specific brain regions seem to be more sensitive to ischemic brain injury than others, according to the presence or absence of specific vulnerable neurons populations (White *et al.*, 2000). As an example, CA1 pyramidal neurons of hippocampus are especially vulnerable (Bartsch *et al.*, 2015), while neurons of the brainstem are relatively resistant (Friberg and Cronberg, 2013).

1.1.2. Interventions and treatment

In this context, the time to return of spontaneous circulation (time to ROSC) and in particular the duration of no-flow (time between cardiac arrest onset and start of external cardiac massage) is of critical importance to determine insult severity (Oddo *et al.*, 2008; Wallmüller *et al.*, 2016). The first rhythm detected by the emergency team is also informative for assessing chances of survival: ventricular fibrillation, indicating a disorganized cardiac

activity, is associated with better outcome as compared to asystole (no cardiac activity detectable) or pulseless electrical activity (electrical cardiac impulse not followed by muscular contraction) (Oddo *et al.*, 2008).

The complexity of the multiple pathophysiological mechanisms entailed as a consequence of global cerebral ischemia makes it difficult to find a unique therapy targeting one specific aspect of these processes (Taccone *et al.*, 2015). Up to now, the only intervention that has shown significant results is targeted temperature management, where body temperature is decreased, kept at 33°-36°C for 12-24 hours. The exact mechanisms of neuroprotection through temperature lowering are still not completely understood, but some evidence show that it acts on several processes of the secondary injury, such as decreasing free radical production and propagation, decreasing lipolysis, inducing pro-survival signalling processes, etc. (White *et al.*, 2000). The introduction of therapeutic hypothermia (TH; see Fig. 1) as a standard of care after cardiac arrest displayed promising results for improving survival rate (Bernard *et al.*, 2002; Oddo *et al.*, 2006), as well as the cognitive outcome of survivors (Tiainen *et al.*, 2007). However, recent studies challenged these initial conclusions by showing no difference in survival between patients treated with therapeutic hypothermia and patients in controlled temperature condition (36°C) (Nielsen *et al.*, 2013). In the same direction, there were no significant difference in the cognitive and functional outcome of both survivors groups (Cronberg *et al.*, 2015; Lilja, Nielsen, *et al.*, 2015).

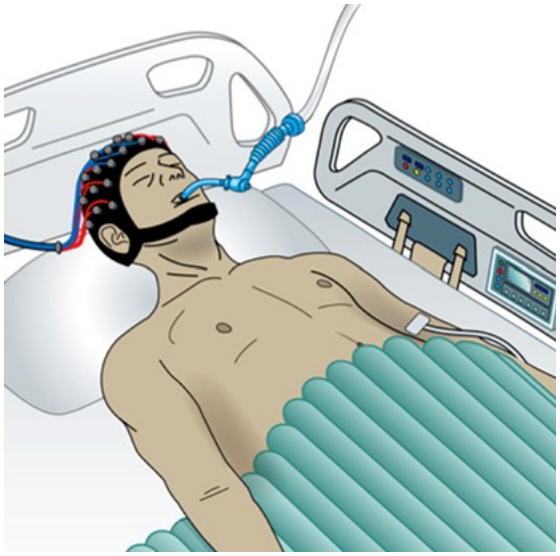

Fig. 1: Exemplar clinical setting in post-resuscitative care after cardiac arrest. Comatose patients are intubated (blue tube), and covered by a cooling device controlling temperature (green blanket). Brain activity can be monitored through electroencephalography (black cap). Adapted from (Taccone *et al.*, 2014).

Resuscitated patients treated with therapeutic hypothermia also receive sedative drugs and neuromuscular blockers. Hypothermia impairs drug metabolism and hepatic and renal

clearance, resulting in high concentrations of sedative agents even several hours after discontinuation of medication (Greer *et al.*, 2014). Importantly, sedation and hypothermia can significantly change markers of recovery during coma, which in turn might alter and delay outcome prediction (Samaniego *et al.*, 2011; Tortorici *et al.*, 2007).

1.1.3. Investigating brain functions

The complete loss of awareness of the environment and the impairment of arousal characteristic of coma makes the clinical exploration of cerebral integrity difficult to assess. Currently, in clinical practice several tests are used to evaluate different parameters of brain preservation in comatose patients. In this section, I will briefly present the clinical tests routinely performed and their prognostic value; then I will describe in further details the electrophysiological investigations of particular interest for my thesis topic.

Neurological examination, testing brainstem reflexes and motor response to noxious stimuli, is fast to perform and provides valuable prognosis information. Brainstem structures are relatively resistant to anoxia and can recover at least partially within the first hours after cardiac arrest, even in patients with severe hypoxic-ischemic injury (Friberg and Cronberg, 2013). Absent brainstem reflexes, including pupillary, corneal and oculo-cephalic reflexes, 72h after coma onset is robustly associated with poor outcome in patients treated with therapeutic hypothermia with very low false positive rates (0.5-5%) (Fugate, Wijdicks, *et al.*, 2010; Rossetti *et al.*, 2016; Al Thenayan *et al.*, 2008). Motor response reflects the integrated function of brainstem and higher cerebral structures and is usually classified as good (flexion response to pain or more directed motor response) or poor (extension response to pain or no motor response). No recovery of motor response 3 days after coma onset is associated with poor outcome, however with important exceptions (false positive rate: 10-24%) (Fugate, Wijdicks, *et al.*, 2010; Rossetti *et al.*, 2016; Rossetti, Oddo, *et al.*, 2010). The presence of myoclonus, indicating cortically-generated epileptic seizures or a profound brainstem dysfunction manifesting through intense involuntary shaking, is another clinical marker of the brain condition in comatose patients. The occurrence of myoclonus early in the time-course of post-anoxic coma is strongly associated with poor prognosis (false positive rate: 0-11%; chances of survival increase if the EEG is reactive and continuous and brainstem reflexes are visible) (Fugate, Wijdicks, *et al.*, 2010; Rossetti *et al.*, 2016; Thömke *et al.*, 2005).

More recently, specific blood biomarkers of neuronal integrity have been introduced in the routine clinical practice, such as the neuron-specific enolase (NSE) and the S-100B protein. NSE is indicative of neuronal death, while the S-100B protein indicates micro-glial damage (Rossetti *et al.*, 2016). In post-anoxic coma, a high S-100B value on the third day of coma is associated with worse cognitive performance at 6 months (Prohl 2007) and a NSE value above 33µg/l is strongly associated with poor outcome but with a high false-positive rate (up to 30%) (Cronberg *et al.*, 2011; Fugate, Wijdicks, *et al.*, 2010). A recent study showed that

NSE provides reliable outcome information at 48h and 72h after cardiac arrest (Stammet *et al.*, 2015). However, in the absence of widely established cut-off values, these biomarkers are currently used rather as additional information.

Electrophysiology

Electroencephalography (EEG) is a non-invasive electrical neuroimaging technique allowing the investigation of brain activity without the active participation of the individual. The signal is collected through electrodes placed on the scalp and reflects the synchronized action potentials of pyramidal neurons oriented perpendicularly to the surface (Brandeis *et al.*, 2009). EEG is widely used for the investigation of many neurological diseases such as epilepsy and disorders of consciousness, as well as in research settings for its several advantages. Indeed, EEG is a portable and low cost tool, with an excellent temporal resolution combined with a good spatial resolution for high-density montages (Michel *et al.*, 2004). In particular, EEG is extremely valuable for the investigation of brain condition in non-responsive patients, even in hypothermic conditions. Therefore, it is recommended for the clinical evaluation of post-anoxic comatose patients since the 1960s (Hockaday *et al.*, 1965).

In this specific context, clinical EEG is most often described along three dimensions providing valuable information to the prediction of patients' outcome: (1) background activity, (2) reactivity to stimuli and (3) epileptiform patterns (Rossetti *et al.*, 2012).

The first dimension, background activity, describes the spontaneous brain signal in terms of continuity, i.e. the regularity of the cerebral activity along the duration of the recording. Established criteria (Hirsch *et al.*, 2013) are used to categorize background activity from "continuous" (when the brain activity is not interrupted by any periods of attenuation of the signal), "discontinuous" (attenuation between 10%-50%), "burst-suppression" (when more than 50% of the signal is attenuated) to "suppressed" (when voltage is persistently below 10 μV). Several studies have reported the continuity dimension as one of the most indicative features of brain preservation in post-anoxic coma: a continuous pattern in normothermia is associated with regaining consciousness with 100% specificity (Rundgren *et al.*, 2006), while burst-suppression has been shown to be related to mortality (Rundgren *et al.*, 2010; Sadaka *et al.*, 2015; Sivaraju *et al.*, 2015) and generalized suppression to no awareness recovery (Cloostermans *et al.*, 2012; Thenayan *et al.*, 2010; Westhall *et al.*, 2016).

The second dimension, reactivity, refers to any reproducible change in amplitude or frequency in the EEG signal, in response to patient stimulation (Rossetti, Oddo, *et al.*, 2010). Reactivity is tested in response to external stimulation, mostly auditory or nociceptive, and is categorized as present or absent. An unreactive EEG background is rarely compatible with good neurological recovery and strongly associated with in-hospital mortality (93% specificity) (Rossetti, Oddo, *et al.*, 2010; Thenayan *et al.*, 2010). Conversely, reactivity to stimuli in the early phase of coma (within 48 hours) has been reported to be strongly

associated with recovery of awareness (94% specificity) (Thenayan *et al.*, 2010) and with survival (Crepeau *et al.*, 2013; Rossetti, Oddo, *et al.*, 2010; Tsetsou *et al.*, 2013).
The third dimension, epileptiform activity, refers to the detection of ictal or interictal features related to epileptic seizures, such as spikes or sharp waves. The most frequently observed patterns are generalized periodic discharges (GPDs; Milani et al., 2014), seizures (Knight *et al.*, 2013; Sivaraju *et al.*, 2015) and status epilepticus, which refers to seizures lasting for more than 5 minutes (Legriel *et al.*, 2013; Rittenberger *et al.*, 2012; Rossetti *et al.*, 2007). In some patients, seizures can be detected during hypothermia and under sedation with antiepileptic general anaesthetics, such as midoazolam or propofol (Crepeau *et al.*, 2013; Mani *et al.*, 2012; Rittenberger *et al.*, 2012). In particular, status epilepticus is relatively common, occurring in 1/3 of resuscitated patients, (Knight *et al.*, 2013; Legriel *et al.*, 2013), and is strongly related to poor outcome, especially when manifesting with early myoclonus (nearly 100% specificity) (Legriel *et al.*, 2013; Rittenberger *et al.*, 2012; Rossetti *et al.*, 2007; Sadaka *et al.*, 2015).
In addition, the combination of these EEG patterns provides accurate prognostic information, as proved by the simultaneous occurrence of burst-suppression, presence of status epilepticus, and lack of reactivity, which seems to be always associated with non-survival (Fugate, Wijdicks, *et al.*, 2010).

Somato-sensory evoked potential (SSEP) is a specific electrophysiological component elicited after a non-invasive stimulation of the median nerve at the wrist or elbow with a small electric current. The complete pathway of the electric stimuli can be assessed from the periphery to the sensory cortex including spinal cord, brainstem and thalamus, through electrodes placed at different locations (Tjepkema-Cloostermans, van Putten, *et al.*, 2015). The N20 is the earliest cortical potential, generated in the primary somatosensory cortex and reflecting the preservation of thalamocortical projections; it is particularly robust and is the latest component to disappear after severe encephalopathy or important sedation (Tjepkema-Cloostermans, van Putten, *et al.*, 2015). In post-anoxic coma, SSEP investigations can be performed very early and provide valuable prognostic information. In particular, absent N20 is related to non-survival with extremely high specificity, and is therefore considered as one of the most reliable predictors of poor outcome (Bouwes *et al.*, 2009, 2012; van Putten, 2012). However, intact N20 response is not indicative of good outcome, as many patients with preserved SSEP may still die, demonstrating the low sensitivity of this test (Greer *et al.*, 2014).

Multimodal evaluation and withdrawal of intensive care support
Each of these clinical tests provides valuable information about brain preservation and prognosis. However, taken separately, none of them is robust enough to give a reliable

estimation of patient's chances of survival (Horn *et al.*, 2014; Rossetti *et al.*, 2016; Sandroni *et al.*, 2014). In order to minimize false prediction, multimodal assessment combining the paramount assessment of clinical examination (brainstem reflexes, motor response, myoclonus), EEG and SSEP is currently recommended (Ben-Hamouda *et al.*, 2014; Friberg and Cronberg, 2013; Fugate, Wijdicks, *et al.*, 2010; Sandroni, Cavallaro, Callaway, D'Arrigo, *et al.*, 2013; Sandroni, Cavallaro, Callaway, Sanna, *et al.*, 2013). As described in Fig. 2, these evaluations should take place 48h after cardiac arrest at the earliest, off sedation and in normothermic conditions, and the clinical status should be repeated at 72h for patients not showing clear signs of awakening (Rossetti *et al.*, 2016). In most cases, these tests are sufficient to orient prognostication (Step 1 in Fig. 2), but in case of doubt, biochemical markers (NSE in particular; Step 2 in Fig. 2) and cerebral magnetic resonance imaging (MRI; Step 3 in Fig. 2) can provide confirmatory information.

In order to decide the interruption of intensive life support with the lowest risk of false pessimistic predictions, at least two of the aforementioned paramount tests should indicate clear signs of poor outcome (Rossetti *et al.*, 2016; Sandroni *et al.*, 2014).

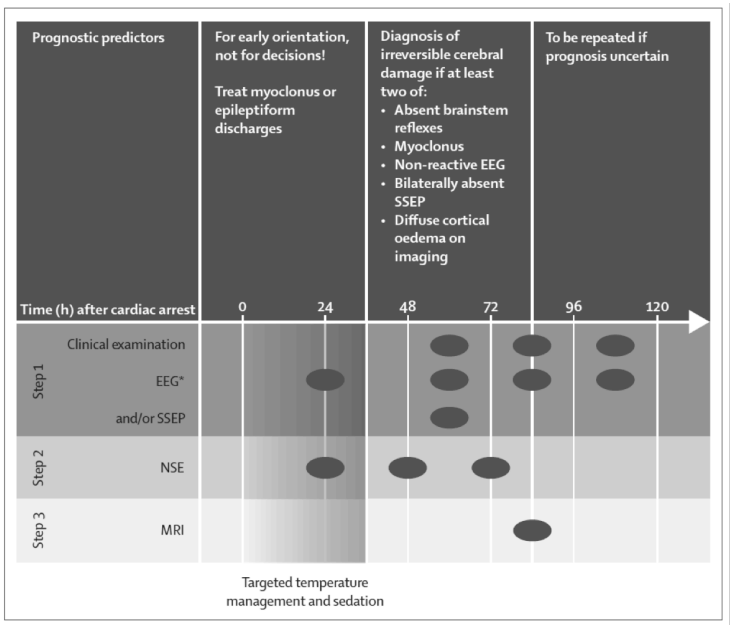

Fig. 2: Recommended multimodal algorithm for outcome prediction after cardiac arrest. Adapted from (Rossetti *et al.*, 2016).

Clinical scales

Typically, recovery after cardiac arrest follows a specific pattern. The first brain region to recover is the brainstem, followed by subcortical structures, and eventually the cortex, with consciousness recovery (Friberg and Cronberg, 2013). In order to assess the severity of coma and the progressive recovery of brain functions, as well as to communicate accurately between medical caregivers, some clinical scales have been developed. The most commonly

used is the Glasgow Coma Scale (GCS) (Teasdale and Jennett, 1974), which evaluates three dimensions of responsiveness on a total of 15 points: eye (4 points), motor (6 points) and verbal response (5 points). Although the GCS is widely used in emergency settings, it has limitations when it comes to be used in more chronic situations. In particular, considering that comatose patients are intubated, the verbal response cannot be tested. In addition, the GCS does not take into account clinical indicators of severity of coma such as brainstem reflexes and breathing patterns, thus failing in detecting changes in neurological examination. To address these issues, a newer scale has been proposed, the Full Outline of Unresponsiveness (FOUR) (Wijdicks *et al.*, 2005). This evaluates four dimensions: eye response, motor response, brainstem reflexes and respiration; each is rated on a 4 points scale, resulting in a maximum of 16 points. This scale is simple and rapid to use, has demonstrated excellent interrater reliability, and is applicable in a wide range of acute neurological conditions. Moreover, it provides prognosis information as a low FOUR score is related with increased mortality (Fugate, Rabinstein, *et al.*, 2010; Wijdicks *et al.*, 2005).

Auditory evoked potentials

Along with clinical investigation of EEG patterns, the investigation of EEG changes in response to incoming sensory stimuli provides reliable insights about the preservation of information processing in cortical and sub-cortical circuits (Brandeis *et al.*, 2009). Event-related potentials (ERPs) refer to the brain responses to any sensory stimuli attested as amplitude modulations with respect to baseline. Because the amplitude modulation elicited by the stimulus occurs at very low voltage (2-10μV), ERPs analyses are classically performed by averaging a large number of trials to obtain a reliable signal.

Auditory evoked potentials (AEPs) refer specifically to ERPs elicited by auditory stimuli. AEPs components are typically named accordingly to the polarity (negative vs. positive) and the latency of the peak (in milliseconds) as measured after averaging hundreds of peri-stimulus AEPs responses in healthy controls. For example, the N100 component is characterized by a negative polarity in fronto-central electrodes and elicited at ~100 ms after the auditory stimulus onset. Another common AEP component is the mismatch negativity (MMN), peaking at ~150-250 ms after stimulus onset in fronto-central electrodes (Garrido *et al.*, 2009). The MMN is a pre-attentive component, occurring when a rare deviant sound interrupts a sequence of frequent standard sounds (Näätänen *et al.*, 1978). Therefore, it reflects the neural response to regularity-violation detection and can be used as an indicator of auditory discrimination between two different categories of stimuli (Näätänen *et al.*, 2007). Classically, the MMN component is visualized by subtracting the AEPs of the deviant sounds by the AEPs of the standard sounds at frontal locations, showing the typical negative shift in brain potential at around 150 ms (Fig. 3 Panel d).

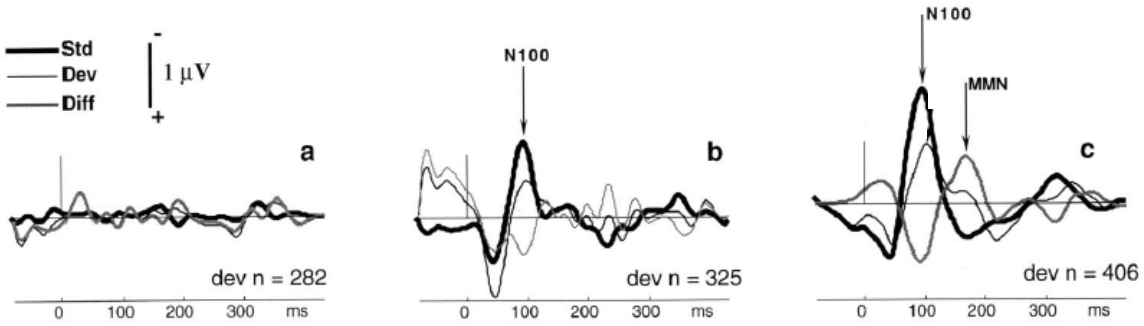

Fig. 3: Average AEPs at frontal electrodes in response to standard and deviant sounds recorded from three comatose patients during the presentation of a MMN paradigm. (a) Patient showing no sign of evoked activity to the stimuli (both N100 and MMN components are absent); (b) Patient showing an evoked response (N100) without MMN component; (d) Patient showing both N100 and MMN components. Modified from (Fischer et al., 1999).

As the mismatch negativity is elicited without requiring the active participation of the individual, MMN paradigms have been widely used in comatose patients to assess the cerebral integrity of auditory functions (Daltrozzo *et al.*, 2009). Interestingly, the presence of a MMN component in comatose patients is strongly associated with awakening from coma, providing valuable prognosis information (Fischer *et al.*, 2004; Naccache *et al.*, 2005; Wijnen *et al.*, 2007).

However, these evaluations were performed at various delays after coma onset and in patients with diverse clinical etiologies. Moreover, the classical investigations of the MMN component require specific a priori hypotheses in terms of amplitude, latency and electrode locations, which can be severely changed in coma conditions. In addition, the identification of the N100 component to certify the presence of a robust auditory response to sounds is often used as a prerequisite before further exploring the occurrence of the MMN component. This prerequisite is not met for as much as 33% of the patients (see Fig. 3 Panels a and b), thus disregarding an important proportion of the studied population (Fischer *et al.*, 1999). To address these issues, multivariate decoding algorithms taking advantage of EEG voltages topographies across the whole electrode montage have been developed (Tzovara, Murray, Michel, *et al.*, 2012; Tzovara, Murray, Plomp, *et al.*, 2012). By building a prototypical model of the brain topographies elicited for each condition and then quantifying their difference, this method provides a reliable measure of auditory discrimination, and has been proven efficient to distinguish between standard and deviant sounds in comatose patients (Tzovara *et al.*, 2013, 2016). It is especially advantageous in conditions where the EEG signal is highly variable as compared with stable modulations in normal healthy conditions (De Lucia and Tzovara, 2014).

1.1.4. Assessing cognitive and functional outcome

As explained in previous sections, rapid provision of care and most recent treatments have significantly improved survival of comatose patients in the last years (Fugate *et al.*, 2012). However, around half of the patients will not survive to hospital discharge, arising ethical and practical issues about the decision to maintain intensive care in subjects with signs of poor prognosis. In this context, the assessment of the quality of survival is extremely important to increase the appreciation on the risk of survival with severe and disabling cognitive dysfunctions. Neurological recovery can occur up to several weeks after cardiac arrest, although most patients with good neurological outcome will improve within 1-2 weeks after the insult (Horn *et al.*, 2014). Clinical studies report outcome at various delays from the initial insult. Classifying outcome at hospital discharge allows describing patients' clinical condition close to the initial event, while minimizing the influence of external factors; outcome assessment at 3 or 6 months is frequently reported to obtain an evaluation of patients' status after what is assumed to be a nearly complete recovery.

To date, the most commonly used tool to characterize outcome after cardiac arrest is the Cerebral Performance Category (CPC) (Booth *et al.*, 2004). CPC scale ranges from 1 (survival with good recovery, "back to baseline") to 5 (death); CPC 2 indicates moderate cerebral disability, CPC 3 severe cerebral disability with dependency for daily life activity and CPC 4 comatose or vegetative state. CPC 1-2 is usually considered as good, while CPC 3-5 as poor outcome. Using the CPC, a recent study on a very large cohort of cardiac arrest survivors (n = 980) reported a good outcome at hospital discharge in the vast majority (85%) of them (62% CPC 1 and 23% CPC 2), highlighting the relatively low occurrence of CPC 3 and 4 in cardiac arrest survivors (Phelps *et al.*, 2013). When assessed at hospital discharge, a CPC is often informative of long-term survival, as a favorable CPC predicts better prognosis (Kim *et al.*, 2016; Phelps *et al.*, 2013).

The modified Rankin scale (van Swieten *et al.*, 1988) is another tool initially designed for stroke patients (Wilson *et al.*, 2002) and widely used to characterize outcome after cardiac arrest (Horn *et al.*, 2014). Compared to the CPC score, the modified Rankin scale describes outcome on a larger range of categories, providing more detailed information concerning recovery. It describes functional outcome in terms of limitations in activities and changes in lifestyle on a 7 points scale. A score of 0 (no impairment) to 2 points (disability but still independence for daily activities) is generally considered as a good recovery, while scores 3-5 involve dependency to others and 6 indicates death. Using the modified Rankin Scale to characterize recovery 6-12 months after cardiac arrest, a recent study showed that 81% of the survivors had a good recovery (Beesems *et al.*, 2014).

The CPC and the modified Rankin scales provide thus rapid outcome assessment, however they are quite limited for a more refined cognitive and functional evaluation. Indeed, when assessing long-term cognitive outcome with slightly more specific tools (i.e. using a short telephone interview), a recent study showed that up to 40% of the survivors experience some kind of cognitive impairment 20 months after cardiac arrest (Fugate *et al.*, 2013). In addition, a systematic review highlighted that cognitive dysfunction can be observed in about 50% of survivors at long-term follow-up when using neuropsychological testing targeting specific cognitive functions (Moulaert *et al.*, 2009). The majority of affected cognitive domains relate to impairment of long-term memory, attention and executive functioning, and to a lesser extent fine-motor functioning (van Alem *et al.*, 2004; Alexander *et al.*, 2011; Cronberg *et al.*, 2009; Ørbo *et al.*, 2014; Peskine *et al.*, 2010). Compared to matched healthy controls, cardiac arrest survivors show impaired long-term memory dysfunction, worse working and prospective memory but preserved recognition and short-term memory (Sulzgruber *et al.*, 2015). Contrary to a widespread idea, isolated amnesia is rare after cardiac arrest and the occurrence of memory deficits often coexist with other cognitive impairments (Lim *et al.*, 2004). Functional disability has also been reported in several studies, with cardiac arrest survivors exhibiting quality of life impairment, emotional problems such as anxiety and depression, or fatigue (Beesems *et al.*, 2014; Hofgren *et al.*, 2008; Larsson *et al.*, 2014; Moulaert *et al.*, 2010; Wachelder *et al.*, 2009).

Some studies suggested that therapeutic hypothermia treatment could also improve cognitive outcome on top of increasing survival (Ørbo *et al.*, 2014; Tiainen *et al.*, 2007). However, the latest studies on very large samples comparing long-term cognitive outcome of patients treated with hypothermia with patients receiving controlled temperature management at 36°C showed no significant difference between the two groups and confirmed the presence of cognitive impairment in about 50% of the survivors (Lilja, Nielsen, *et al.*, 2015; Tiainen *et al.*, 2015). Quality of life was also good and similar in both groups, even though a substantial proportion of patients (around 40%) reported that they did not achieve a full recovery (Cronberg *et al.*, 2015). Up to 24% of the survivors also experienced anxiety, but depression was not very common (13%) (Lilja, Nilsson, *et al.*, 2015).

As exposed, in the absence of guidelines for assessing cognitive functions, outcome assessment varies widely among studies, ranging from one single test or a combination of tests incorporated in one scale (e.g. Mini-Mental State examination) to an exhaustive neuropsychological assessment (Moulaert *et al.*, 2009). Combined with the lack of consensus regarding specific thresholds for categorizing good and bad outcome, these findings are difficult to compare and integrate. In particular, cognitive disability is often underestimated because sensitive measures are not systematically applied, only few high-quality prospective studies have been realized and some impaired patients might be disregarded because of their inability to participate to a long-term follow-up assessment.

1.2. Evolution of EEG reactivity from hypothermia to normothermia

The fact that EEG examination can be performed at any moment during the time course of coma opens the possibility of evaluating the evolution of cerebral functions. Indeed, EEG signal can reflect the initial cerebral insult within the first hours after cardiac arrest (Alvarez *et al.*, 2013; Hofmeijer and Van Putten, 2012). Later in time, the persistence of pathological patterns may signal a severe injury, while normalization is suggestive of an on-going recovery process that might ultimately lead to awakening (Crepeau *et al.*, 2013). **In this context, evaluating the evolution of EEG brain signal over time is important to assess the recovery of brain functions and patients' chances of survival**.

In order to witness this evolution, EEG assessments can be performed at separated occasions or in a continuous fashion over the time course of coma. For the former option, a common approach is to record the first time within 24h after coma onset (but at least 9-12h afterwards) under TH, and the second time at 48h and after return to normothermic conditions. Sedation typical of targeted temperature management on the first day of coma may affect EEG, depending on dosages (Crepeau *et al.*, 2015). Therefore, current guidelines recommend to use only the EEG assessment in normothermia and off sedation as a prognostic tool (Cronberg *et al.*, 2013; Greer *et al.*, 2014). However, recent studies suggested that some EEG patterns can be informative of prognosis already during TH and that EEG patterns in TH have similar value as in normothermia (Cloostermans *et al.*, 2012; Crepeau *et al.*, 2013, 2015; Rossetti *et al.*, 2012). In particular, the presence of epileptiform transients and lack of reactivity seems to be a robust predictor of poor outcome in TH (Oddo and Rossetti, 2014; Rossetti *et al.*, 2012; Rossetti, Urbano, *et al.*, 2010).

EEG reactivity to stimuli seems to be a reliable marker of brain recovery and a robust outcome predictor (Oddo and Rossetti, 2014; Thenayan *et al.*, 2010). A previous study showed that a reactive EEG pattern during TH was strongly associated with survival, especially if the EEG remained reactive after rewarming (Tsetsou *et al.*, 2013); moreover, loosing reactivity in normothermia seems to be rare, and invariably associated with non survival. However, whether a non-reactive EEG in TH is reliably predictive of poor outcome independently from its evolution over time remains unknown.

In this first study, I focused specifically on patients showing a non-reactive EEG in TH. **The first aim was to investigate whether a non-reactive EEG pattern in TH might be used as a reliable marker of poor outcome.** If this was the case, it would provide an indication of prognosis that would be available very early in the time course of coma. **The second aim was to observe the evolution of a non-reactive hypothermic EEG to normothermic conditions in relation to complementary clinical examinations.** In particular, I wanted to

investigate whether the return of EEG reactivity in normothermia would be associated with recovery of other brain functions. To these objectives, I collected all clinical examinations (neurological assessment, NSE samples, SSEP exams, etc.) for all post-anoxic comatose patients exhibiting a non-reactive EEG in TH and having a second EEG recording in normothermia. Then, I compared outcomes and results to these clinical tests of patients recovering EEG reactivity in normothermia to those remaining non-reactive.

1.3. Prognostication of cognitive and functional outcome

Cognitive dysfunction is common in cardiac arrest survivors, and ranges from mild to severe impairment. These troubles can have an important impact in patients' daily life functioning and subjective quality of life. However, to date, very little is known about the prediction of detailed outcome of the survivors. **The identification of reliable predictors of cognitive dysfunctions very early in the time course of coma would provide critical information of brain recovery.** Being able to predict the expected cognitive status of each patient could help the clinical teams to more accurately guide treatments early after the initial insult in the objective of improving patients' outcomes, and to reliably orient relatives.

Environmental and demographics predictors

The first studies trying to predict cognitive and functional outcome after cardiac arrest investigated the impact of environmental or demographic variables. In particular, etiology of cardiac arrest and age at the event were associated with quality of life and cognitive functions at 3 months follow-up (de Vos *et al.*, 1999). Resuscitation parameters such as heart massage starting time, time to first shock, or duration of cardiac arrest can be considered as indicators of ischemia duration and therefore used to predict brain insult severity. Whether these time-related elements have an impact on cognitive outcome of cardiac arrest survivors is debated**.** Some studies reported no relationship between duration of resuscitation and cognitive functions (de Vos *et al.*, 1999), while others rather pointed out an inverse correlation between duration of cardiac arrest and memory scores (Grubb *et al.*, 1996). Using more detailed cognitive outcome measures at 6 months, van Alem et al. (2004) showed that receiving cardio-pulmonary resuscitation before ambulance arrival was related to better cognitive performance, especially in immediate recall and visuomotor tracking (van Alem *et al.*, 2004). However, time to cardiopulmonary resuscitation, time to first shock or duration of cardiac arrest were not associated with preserved cognitive functions (van Alem *et al.*, 2004). Furthermore, more recent studies demonstrated that neither age or duration of cardiac arrest were associated with cognitive outcome (Fugate *et al.*, 2013; Ørbo *et al.*, 2014). In conclusion, resuscitation time, demographic variables and medical comorbidities showed inconclusive results regarding their impact on cognitive outcome.

In contrast, coma duration has been reliably associated with functional outcome: patients with short coma duration have less complaints of cognitive functioning and higher quality of life (Middelkamp *et al.*, 2007). More recent studies confirmed these results by showing that shorter coma duration and induced hypothermia treatment were associated with better cognitive outcome (Alexander *et al.*, 2011; Ørbo *et al.*, 2014), suggesting strong relationship between coma duration and recovery of brain functions. However, considering that coma duration can be measured only after awakening, this variable provides limited information.

Neurological predictors

Clinical variables available early in the time-course of coma – such as EEG features or brainstem reflexes – are essentially predictive of poor outcome (i.e. death); using them to predict functional recovery of the survivors is therefore extremely challenging (Horn *et al.*, 2014; Sandroni *et al.*, 2014). To date, only one group succeeded to show reliable associations between early investigations during coma and long-term neuropsychological outcome. Considering all clinical variables available early after cardiac arrest, they showed that S-100B measured on day 3 after admission and long-latency somato-sensory evoked potential (N70) on day 4 were associated with selected cognitive variables at 6 months (Prohl *et al.*, 2007). In particular, S-100B was significantly correlated with learning/memory performances and selective executive functions (Prohl *et al.*, 2007, 2009), while N70 was associated specifically with executive performances (Prohl *et al.*, 2007). In addition, a short bedside neuropsychological screening performed within one month after cardiac arrest was the best predictor of long-term cognitive outcome (Prohl *et al.*, 2007, 2009). Despite being very encouraging, these studies are very rare, results have not been systematically replicated, and no cut-off values have been set for a prediction at the single-patient level.

Auditory evoked potentials predictors

As described in a previous section, cognitive auditory evoked potentials, and in particular the MMN component, are the only brain evaluations available early after coma onset able to predict positive outcome, i.e. survival (Daltrozzo *et al.*, 2007; Fischer *et al.*, 2004). The attempts to use AEPs for the forecast of more detailed outcome has been very rare and gave only information for the transition from coma to vegetative state (Fischer and Luaute, 2005; Wijnen *et al.*, 2007). To date, no study used AEPs during coma to predict detailed cognitive and functional outcome of the survivors. In addition, the evolution of the brain responses to such stimuli over time has been poorly investigated (Wijnen *et al.*, 2007).

Recent work demonstrated that the evolution of auditory discrimination early after coma onset was specifically related with survival at three months (Tzovara *et al.*, 2013, 2016). In this setting, the mismatch negativity response to deviant sounds was not investigated with the classical method identifying the component at one electrode but for the whole brain, taking advantage of all electrodes using multivariate EEG analyses (Tzovara *et al.*, 2013; Tzovara *et al.*, 2012). This single-trial decoding algorithm allows characterizing the prototypical voltage topographies for each category of sounds (i.e. standard vs. deviant sounds) and quantifies their difference. A difference of voltage topographies is indicative of different brain generators (Tzovara, Murray, Plomp, *et al.*, 2012), suggesting different underlying activations for each condition. Results showed that auditory discrimination could be detected even on the first day of coma and under TH, especially in non-survivors.

Survival, however, was not related to the absolute score of auditory discrimination on the first or second day, but rather to its progression over time. Indeed, only survivors exhibited an improvement of auditory discrimination over the two days (Fig. 4) (Tzovara *et al.*, 2013), which was confirmed with only few exceptions in the subsequent validation study, resulting in 93% specificity for predicting survival (Tzovara *et al.*, 2016)**.** This evolution of auditory discrimination specific to survivors could suggest a progressive recovery of brain functions, which will ultimately lead to awakening. However, among patients not showing such an improvement, some will still survive, questioning the mechanism of brain recovery in such cases. Why is the progression of auditory discrimination during coma evident only in a subset of survivors? And is this evolution of auditory discrimination only indicative of survival or does it reflect more generally the recovery of brain functions?

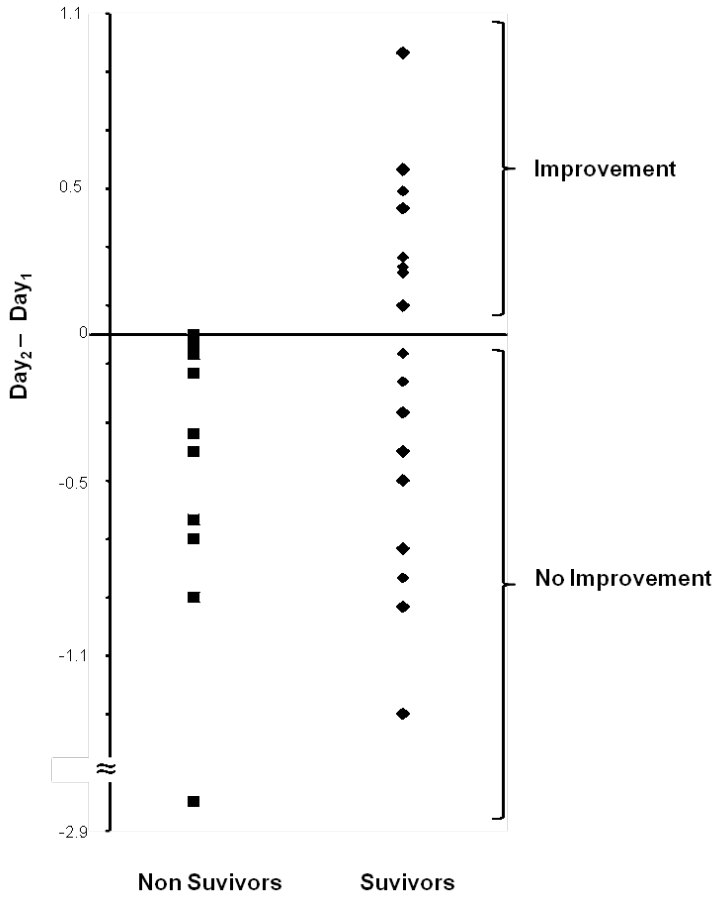

Fig. 4: Difference in auditory discrimination assessed during the second and the first day of coma in Non Survivors (left column) and Survivors (right column): an improvement is observed exclusively in survivors. Adapted from (Tzovara *et al.*, 2013).

In this second study, my aim was to focus specifically on survivors to investigate whether they differ in cognitive and functional outcome according to the evolution of auditory discrimination. **My hypothesis was that an improvement of auditory discrimination during coma is a sign of early recovery of brain functions, and therefore would be**

associated with better cognitive performances on awakening. In this sense, I stated that the progression of auditory discrimination during coma could be not only predictive of survival but also of the detailed cognitive and functional outcome of the survivors. The identification during early coma of survivors who will suffer cognitive deficits and poor recovery would be extremely useful for relatives and clinicians, and could lead to an adaptation of the treatments to improve patient's outcome. Moreover, it would be the first attempt to use the evolution of auditory evoked potentials to predict functional outcome of survivors. To this aim, I recorded 96 post-anoxic comatose patients on the first and second coma day and assessed cognitive functions of survivors using a neuropsychological examination shortly after awakening. Functional outcome was assessed at 3 months after cardiac arrest using the CPC score, categorized as excellent (CPC 1) vs. moderate (CPC 2-3) recovery. In addition, I also considered additional variables indicative of clinical recovery, such as the duration of coma and overall hospital stay or the indication to a specialized in-patient neurorehabilitation.

1.4. Cognitive functions in altered states of consciousness

The neural basis of the conscious experience in humans is becoming an increasingly important field of research in cognitive neuroscience. Historically, one of the main research challenges has been the difficulty of introducing a formal and unambiguous definition of consciousness, and to find appropriate experimental strategies for its investigation (Crick and Koch, 1990). At present, the most agreed current definition of consciousness refers to the pure subjective experience that each human individual can report at a given moment (Tononi and Koch, 2015). Typically, consciousness vanishes when we sleep although it can occur during some conditions such as some forms of dreaming (i.e. lucid dreaming). **While there is little doubt about the conscious state of healthy awake people, inferring consciousness in specific clinical conditions such as coma, vegetative state, or minimally conscious state is challenging.** These states can be characterized along two main dimensions, i.e. the level of consciousness (wakefulness or arousal) and the content of consciousness (awareness or experience; see Fig.5) (Laureys *et al.*, 2009).

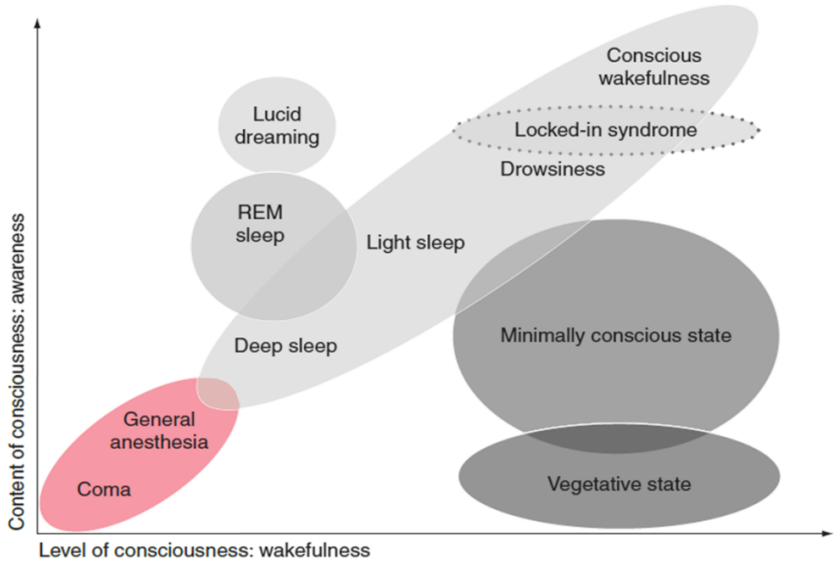

Fig. 5: Distribution of consciousness states according to the two dimensions of consciousness. Coma and general anesthesia are the states with the less of both content and level of consciousness. Adapted from (Laureys *et al.*, 2009).

In order to assess consciousness in these unresponsive patients, single individuals can be tested through specifically designed experimental paradigms. Typically such paradigms target the identification of “markers of consciousness” in the neural activity of healthy controls, for which the conscious experience can be assessed by explicit subjective reports. Subsequently the occurrence of such marker is assessed in patients with disorders of consciousness (DOC). Some experimental tests for the assessment of consciousness in DOC patients have been inspired by the similarity between the neural activity of an imagined

specific movement and those occurring when the actual movement is performed. In one popular functional magnetic resonance imaging (fMRI) paradigm, subjects are asked to imagine either to spatially navigate their house or to play tennis (Monti *et al.*, 2010; Owen *et al.*, 2006). The activation of the specific neural networks underlying these two imagery tasks can be used to reliably associate volitional brain activity and 'yes/no' answer in communication protocols without verbal reports. This paradigm was successfully implemented for assessing consciousness both in healthy individuals (Boly *et al.*, 2007) and vegetative state patient (Monti *et al.*, 2010; Owen *et al.*, 2006). Along the same line, EEG correlates to command following (i.e. moving toes vs. hand) also demonstrated evidence of covert awareness in some vegetative state patients (Cruse *et al.*, 2011), suggesting residual awareness and cognition.

Another approach to assess the presence of consciousness is to take advantage of those tasks that cannot prescind from conscious processing at least in healthy controls. In particular, the electrophysiological detection of novel auditory stimulus in series of standard events includes an early pre-attentive, automatic and non-conscious response, the mismatch negativity, and a late attentive component, the P300, which has been related to conscious awareness of the stimulus (Sergent *et al.*, 2005). In order to isolate the occurrence of the attentive P300 from the automatic MMN, Bekinschtein et al. (2009) designed a paradigm testing auditory regularities within two embedded levels, i.e. local (within trials) and global (across trials) level (Bekinschtein, Dehaene, *et al.*, 2009). Violations of local regularities should elicit the automatic MMN component irrespective of the consciousness state of the individual, while violations of global regularities should elicit the P300 and be detected only in conscious individuals. As expected, all patients exhibited an effect to local deviants, while only conscious individuals detected global violations, confirming that only patients with residual consciousness can demonstrate reliable evidence of the maintenance of perceptual representations across time (Bekinschtein, Dehaene, *et al.*, 2009). However, recent results challenged this claim by showing that some vegetative state patients (King *et al.*, 2013) and more impressively, some deeply comatose patients (Tzovara *et al.*, 2015), can demonstrate reliable evidence of detection of global deviants. The progression of global auditory discrimination over time noted in some survivors (Tzovara *et al.*, 2015) suggests that global discrimination ability could indicate the improvement of specific neural processes underlying the detection of violation detection (as also shown in Tzovara et al 2013) rather than consciousness (Piarulli *et al.*, 2015). Several other studies in healthy and conscious individuals argue against the specificity of the P300 as a marker of consciousness (Koch *et al.*, 2016). In particular, the late component P3b was observed in healthy participants while presenting subliminal stimuli (Silverstein *et al.*, 2015), but not for task-irrelevant stimuli (Pitts

et al., 2012) even when consciously perceived (Pitts *et al.*, 2014). For these reasons, the P3b could in general depend on subjects' attention rather than reflect conscious processing.

Another cognitive process supposed to require consciousness is the one taking place while a new association is formed between two stimuli separated by a temporal gap. This is called trace conditioning (Clark and Squire, 1998), which is usually tested behaviorally by assessing whether the automatic response to an unconditioned stimulus (e.g. startle reflex in response to a loud alarm) can be associated with the occurrence of an initially neutral stimulus preceding the unconditioned stimulus. Few previous studies showed that some DOC patients were able to elicit conditioned responses in an eyeblink trace conditioning procedure (Bekinschtein, Shalom, *et al.*, 2009). However, for all of them, some residual consciousness could not be formally excluded. Another study demonstrated successful trace conditioning in deep sleep by showing significantly higher sniff responses after the presentation of a tone usually paired with a pleasant odor as compared with tones associated with unpleasant odors (Arzi *et al.*, 2012). However, the level of consciousness of these healthy participants was based only on the EEG global sleep scoring, overlooking possible microwake-like activity patterns (Dang-Vu *et al.*, 2008; Nir *et al.*, 2011). In conclusion, previous studies have essentially suggested that trace conditioning can occur in the absence of consciousness; however, evidence of trace conditioning in a deeply unconscious state is lacking, as well as the investigation of the neural correlates underlying the formation of such an association.

The goal of this third project was to investigate the possibility of learning new associations in deeply unconscious conditions based on a trace conditioning paradigm, and whether evidence of this association can be revealed at the neural level. To this aim, I designed an auditory trace conditioning paradigm repeatedly presenting a neutral tone in association with an alerting sound, and recorded the EEG responses to these stimuli in acute comatose patients. I tested learning by comparing the EEG activity elicited by the alerting sound to the EEG activity when the alerting sound was not presented but expected after the tone. This analysis provides robust and specific evidence about learning of the association by testing the response at the exact moment where a precise stimulus is expected. These results would help understand the range of cognitive processing possible without consciousness.

2. SUMMARY OF THE RESULTS

2.1. Clinical evolution after a non-reactive hypothermic EEG following cardiac arrest

Elsa Juan, Jan Novy, Tamarah Suys, Mauro Oddo, and Andrea O. Rossetti

Neurocritical Care (2015) 22:403-408

Contribution: elaborated study design, collected and analyzed data, wrote the paper.

Abstract

Background: Lack of electroencephalography (EEG) background reactivity during therapeutic hypothermia (TH) has been associated with poor outcome in post-anoxic comatose patients. However, decision on intensive care withdrawal is based on normothermic (NT) evaluations. This study aims at exploring whether patients showing recovery of EEG reactivity in NT after a non-reactive EEG in TH differ from those remaining non-reactive.

Methods: Patients with non-reactive EEG during TH were identified from our prospective registry of consecutive comatose adults admitted after successful resuscitation from CA between April 2009 and June 2014. Variables including neurological examination, serum neuron-specific enolase (NSE), procalcitonin, and EEG features were compared regarding impact on functional outcome at 3 months.

Results: Seventy-two of 197 patients (37 %) had a nonreactive EEG background during TH with thirteen (18 %) evolving towards reactivity in NT. Compared to those remaining non-reactive (n = 59), they showed significantly better recovery of brainstem reflexes ($p < 0.001$), better motor responses ($p < 0.001$), transitory consciousness improvement ($p = 0.008$), and a tendency toward lower NSE ($p = 0.067$). One patient recovering EEG reactivity survived with good functional outcome at 3 months.

Conclusions: Recovery of EEG reactivity from TH to NT seems to distinguish two patients' subgroups regarding early neurological assessment and transitory consciousness improvement, corroborating the role of EEG in providing information about cerebral functions. Understanding these dynamic changes encourages maintenance of intensive support in selected patients even after a non-reactive EEG background in TH, as a small subgroup may indeed recover with good functional outcome.

2.2. Prediction of cognitive outcome based on the progression of auditory discrimination during coma

Elsa Juan, Marzia De Lucia, Athina Tzovara, Valérie Beaud, Mauro Oddo, Stephanie Clarke, and Andrea O. Rossetti

Under review

Contribution: elaborated study design, collected and analyzed data, wrote the paper.

Abstract

Objective: If predicting survival in post-anoxic comatose patients is challenging, predicting cognitive outcome of those who will survive is extremely difficult. A progression of auditory discrimination in acute coma indicates survival with high specificity, however whether the degree of improvement matters remains unknown. Here we aim at using this test to predict long-term cognitive and functional outcome of comatose patients.

Methods: We prospectively recorded electroencephalography responses to auditory stimuli of post-anoxic comatose patients on the first and second day after admission. For each recording, auditory discrimination was quantified and its evolution over the two recordings was used to classify survivors as "predicted" when auditory discrimination increased vs. "other" if not. Cognitive functions were tested on awakening and functional outcome was assessed at 3 months using the Cerebral Performance Categories (CPC) scale.

Results: Thirty-two patients were included, 14 "predicted survivors" and 18 "other survivors". Compared to "other survivors", "predicted survivors" exhibited a better cognitive status (ability to follow a standardized neuropsychological battery: 86% vs. 44%; Fisher's exact test p = 0.03) and were more likely to show an excellent functional outcome at 3 months (CPC 1: 86% vs. 33%; Fisher's exact test p = 0.004). Moreover, progression of auditory discrimination during coma was strongly correlated with cognitive performance on awakening (phonemic verbal fluency: r_s = 0.48; p = 0.009).

Interpretation: Progression of auditory discrimination during coma predicts cognitive outcome. The degree of improvement is informative of the degree of functional impairment. This test provides early indication of future recovery.

2.3. Evidence of trace conditioning in comatose patients revealed by the reactivation of EEG responses to alerting sounds

Elsa Juan, Nathalie Ata Nguepnjo Nguissi, Athina Tzovara, Dragana Viceic, Marco Rusca, Mauro Oddo, Andrea O. Rossetti, and Marzia De Lucia

In revision

Contribution: elaborated study design, collected and analyzed data, wrote the paper.

Abstract

Trace conditioning refers to a learning process which occurs after repeated presentation of a neutral conditioned stimulus (CS+) and a salient unconditioned stimulus (UCS) separated by a temporal gap. Recent studies have reported that trace conditioning can occur in humans in reduced levels of consciousness by showing a transfer of the unconditioned autonomic response to the CS+ in healthy sleeping individuals and in vegetative state patients. However no previous studies have investigated the neural underpinning of trace conditioning in the absence of consciousness in humans.

In the present study, we recorded the EEG activity of 29 post-anoxic comatose patients while presenting a trace conditioning paradigm using neutral tones as CS+ and alerting sounds as UCS. Most patients received therapeutic hypothermia and all were deeply unconscious according to standardized clinical scales. After repeated presentation of the CS+ and UCS couple, learning was assessed by measuring the EEG activity during the period where the UCS is omitted after CS+ presentation. Specifically we assessed the 'reactivation' of the neural response to UCS omission by applying a decoding algorithm derived from the statistical model of the EEG activity in response to the UCS presentation.

We found a reactivation of the UCS response in absence of stimulation in eight patients, of which five were under therapeutic hypothermia. Additionally, the reactivation effect was temporally specific within trials since the reactivation manifested primarily at the specific latency of UCS presentation and significantly less before or after this period. Our results show for the first time that trace conditioning may manifest as a reactivation of the EEG activity related to the UCS and even in the absence of consciousness.

3. DISCUSSION

3.1. EEG reactivity during TH

In this first study, I assessed the clinical evolution of patients showing a non-reactive EEG in hypothermia. In general, the absence of reactivity in hypothermia is linked to an extremely poor outcome, as confirmed by the present results showing that only one patient out of the 72 included survived at 3 months. Examining the subsequent evolution of EEG reactivity in normothermic conditions, I found that while the majority of patients (82%) continued to display a non-reactive EEG, a subset of them recover EEG reactivity. Based on this evolution, I compared both groups on their clinical evaluations and outcomes. **My results show that the recovery of EEG reactivity in normothermia is an indicator of return of brainstem reflexes, motor responses and level of consciousness, and a tendency towards lower NSE level, suggesting at least partial recovery of brain functions**. Even if these ameliorations were not associated with good outcome, the only survivor of this cohort was a patient recovering a reactive EEG during normothermia, suggesting thin but existing chances of survival in such cases. In the sections below I will discuss the impact of these findings in light of the current literature and show their implications for future studies.

3.1.1. EEG reactivity as a marker of the brain condition

Lack of EEG reactivity might reflect a widespread damage of the ascending reticular activating system, signaling severe impairment of brainstem pathways otherwise resistant to anoxia (Thenayan *et al.*, 2010). As arousal depends on the proper functioning of this ascending reticular activating system, this would explain why awakening seems almost impossible after a non-reactive EEG (Thenayan *et al.*, 2010).

The present study indicates more often preserved brainstem reflexes and motor response in patients showing recovery of EEG reactivity in normothermia. This might be interpreted as a partial recovery of cerebral subcortical and cortical functions, even though the presence of brainstem reflexes and motor response are not in themselves strong indicators of good outcome (Rossetti *et al.*, 2016). This finding is not surprising because clinical EEG evaluation reflects the degree of neuronal injury (Rossetti *et al.*, 2012) and therefore should overlap with other neurological investigations. As compared to brainstem reflexes and motor response, which are impacted by TH and sedation and exert a reliable prognostic value only in normothermia and off sedation (Rossetti *et al.*, 2016), clinical EEG evaluation has the advantage to be informative of prognosis already during TH (Hofmeijer, Beernink, *et al.*, 2015; Rossetti *et al.*, 2012; Tjepkema-Cloostermans, Hofmeijer, *et al.*, 2015). Therefore, one could suggest using EEG reactivity in TH as a reliable substitute of the neurological examination for the investigation of brain condition early after coma onset.

In addition, the tendency towards higher NSE peak values in patients remaining with a non-reactive EEG reactivity is consistent with previous literature (Rossetti *et al.*, 2012), which showed NSE correlates strongly with EEG abnormalities in TH, in particular reactivity and continuity patterns, but less with epileptiform discharges. As NSE indicates structural neuronal injury (Hans *et al.*, 1993), our and other studies suggest that EEG abnormalities early after cardiac arrest associated with high NSE values are not a transient sign of post cardiac arrest dysfunction, but rather a marker of permanent postanoxic injury in patients with non-reactive EEG (Rossetti *et al.*, 2012)

3.1.2. Influence of hypothermia and sedation

Even though some chemical processes such as neurotransmitter release are temperature dependent, the loss of few degrees in mild therapeutic hypothermia has limited effects on the EEG signal (Hofmeijer and Van Putten, 2016). Moreover, if it is true that anesthetics can modify the EEG trace mostly witnessed by a slowing and amplitude attenuation (Brown *et al.*, 2010), abnormal EEG patterns such as suppressed EEG cannot be induced solely by sedative drugs at dosages routinely used in this clinical setting (Hofmeijer and Van Putten, 2016). EEG patterns induced by relatively low doses propofol or midazolam do not have the same prognosis value as when they appear spontaneously (Hofmeijer, Beernink, *et al.*, 2015; Hofmeijer, Tjepkema-Cloostermans, *et al.*, 2015). In particular, occurrence of a still reactive (burst-)suppression induced by propofol should be rather interpreted as the response of a relatively preserved brain to sedation, as opposed to spontaneous occurrence of non-reactive burst-suppression reflecting a severe brain dysfunction (Hofmeijer *et al.*, 2014; Oddo and Rossetti, 2014). Therefore, these data suggest that the evaluation of EEG reactivity performed during anesthesia and lower body temperature is still a valid estimation of the brain condition.

3.1.3. Sensitivity of non-reactive EEG in TH for predicting poor outcome

Recent studies suggest that the characteristics of early EEG patterns are more related with the time elapsed between cardiac arrest and EEG examination rather than the lowering of body temperature and sedative agents (Hofmeijer, Beernink, *et al.*, 2015). Indeed, lack of EEG continuity, reactivity and presence of epileptiform transients in TH are highly predictive of poor outcome (Rossetti, Urbano, *et al.*, 2010).

In contrast with current guidelines recommending to use EEG as a prognostic tool only 72h after cardiac arrest, off sedation and after return to normothermia (Sandroni *et al.*, 2014), the group from van Putten emphasizes the prognostic value of EEG patterns early after cardiac arrest and during hypothermia (Hofmeijer and Van Putten, 2016; Hofmeijer, Beernink, *et al.*, 2015; Tjepkema-Cloostermans, Hofmeijer, *et al.*, 2015). While the EEG is severely disturbed or even completely absent in the first hours after cardiac arrest, reflecting the massive injury

of cortical synapses (Hofmeijer and Van Putten, 2012), patients with good outcome improve EEG activity at least to a certain extent within the first 24h (Hofmeijer, Tjepkema-Cloostermans, *et al.*, 2015). On the contrary, absence of recovery in that time interval is invariably associated with poor outcome (Hofmeijer *et al.*, 2014; Hofmeijer, Beernink, *et al.*, 2015; Sivaraju *et al.*, 2015), suggesting that the difference between these two populations can be established very early after coma onset. My results go into the same direction by indicating that an improvement in EEG reactivity beyond 24h after cardiac arrest is still related to poor outcome in patients that are not reactive during hypothermia. These data suggest that clinical EEG evaluation under hypothermia and sedation is a reliable prognosis predictor of both good and poor outcome (Hofmeijer, Beernink, *et al.*, 2015; Rossetti *et al.*, 2007; Sivaraju *et al.*, 2015; Tjepkema-Cloostermans, Hofmeijer, *et al.*, 2015) and that it might inform more accurately on the cerebral insult of the anoxic injury as compared to later in time after return to normothermic conditions (Rossetti *et al.*, 2012).

3.1.4. Limitations and future perspectives

The main limitation of this study is the relatively small sample size, and in particular the limited number of patients in the group recovering EEG reactivity in normothermia. This however reflects the reality of clinical practice recruiting in a single center, and the present results are in line with other studies. A limitation to the use of clinical EEG reactivity is the difficulty of having inter-rater agreement (Noirhomme *et al.*, 2014; Westhall *et al.*, 2015), despite clear nomenclature (Hirsch *et al.*, 2013) and intensive training of specialized neurologists. However, in the present setting, the two neurologists who interpreted EEG data collaborated together since a long time, increasing the chance of having similar ratings. In addition, a significant advantage of the present study is that it circumvents the problem of the so called “self-fullfilling prophecy”, as EEG reactivity in hypothermia was not used to decide withdrawal of intensive care support. Since one patient with non-reactive EEH during TH awoke, it remains essential to integrate the whole body of clinical prognostic variables for decision of interruption of intensive care support.

Lack of reactivity in TH seems thus to be more strongly associated with poor outcome as compared with absent reactivity in normothermia, suggesting that early EEG is most informative of the cerebral injury. To test this hypothesis, a further study could use a population of patients showing a reactive EEG in normothermia and compare those having a reactive EEG to those having a non-reactive EEG in TH. Furthermore, as the EEG during TH is together with biological markers the only examination available at that time point, it would be interesting to assess the added value of a normothermic EEG as compared to the other clinical evaluations performed in normothermia.

3.2. Prediction of cognitive and functional outcome

In this second study, my goal was to predict the functional recovery of cardiac arrest survivors using EEG responses to sounds during coma. Based on previous studies showing that an improvement of auditory discrimination over the first two days of coma is predictive of survival, I hypothesized that this progression actually mirrors the recovery of brain functions, which would be confirmed by better outcome. To test this claim, I compared the functional recuperation of the cardiac arrest survivors who improved auditory discrimination during coma vs. the others. **My results indicate that patients improving auditory discrimination during coma perform significantly better on demanding and multi-determined cognitive tests, exhibit more often an excellent functional outcome at 3 months, and show better and faster recuperation**. Based on a simple binary cut-off (improvement vs. no improvement), one can therefore predict the subsequent outcome of each single patient. Interestingly, progression of auditory discrimination significantly correlated with both cognitive scores and rapidity of recovery, suggesting that the dynamic evolution of brain functions during coma is directly associated with the extent of the improvement after awakening. **To the best of my knowledge, this study is the first to succeed in predicting detailed recovery of cardiac arrest survivors based on auditory evoked potentials recorded very early after coma onset**. In the following sections, I will discuss these findings in light of the existing literature and critically expose the advantages and the limitations of the present work.

3.2.1. What is predicted: cognitive performances and functional outcome

The present study identified severe cognitive deficits in one third of cardiac arrest survivors who were unable to follow a full neuropsychological examination shortly after awakening. In addition to these patients, some others exhibited more selective cognitive impairments, mainly regarding attention, executive functions and long-term memory domains. Such profile of deficit is consistent with previous literature in cardiac arrest survivors (for a review see (Moulaert *et al.*, 2009)) but also in patients with myocardial infarction (Lilja, Nielsen, *et al.*, 2015). However, impairments of attention, executive functions and long-term memory domains are also frequently encountered in various neurologic and psychiatric diseases, such as depression (Roca *et al.*, 2015), schizophrenia (Ettinger *et al.*, 2015), or traumatic brain injury (Marsh *et al.*, 2016). It could suggest either that these domains are very sensitive to diverse brain insults or diseases, or, alternatively, that there is a selection bias in the choice of tests. In the present study, I included a large number of tests covering six domains (i.e. language, visual gnosia, praxia, short-term memory, long-term memory, executive functions, and visual attention), which should prevent such a bias and strengthen our findings.

Cognitive testing

In view of the important variability of severity of impairment in the present cohort, the ability to perform the whole neuropsychological assessment was considered as a binary measure of patients' general cognitive condition. In addition, the limited number of available scores across all patients leads to a natural selection of tests for group analysis of cognitive impairments. In particular, it appeared that verbal fluency tests were fast to assess, easy to perform at bedside, and feasible even in patients with severe cognitive dysfunctions. The two additional tests available for most of our patients are both widely used in hospital settings to quickly assess patient's global clinical condition. Digit span forward is a simple measure of attention and short-term memory (Ostrosky-Solís and Lozano, 2006), while orientation to time reflects subject's internal representation of the environment (Peer *et al.*, 2015). The limited available scores represent also an advantage when testing associations with outcome predictors by avoiding the common problem of multiple comparisons classically weakening the power of statistical analyses (Aickin and Gensler, 1996).

Functional assessment

To characterize functional outcome, I categorized the CPC score at 3 months as excellent (CPC 1) vs. moderate recovery (CPC 2-3). This dichotomization is not common in outcome studies, where CPC 1-2 is classically considered as a good, and the rest (CPC 3-5) as poor outcome (Horn *et al.*, 2014; Rossetti *et al.*, 2016), mixing severe functional impairment with vegetative state and death. Considering that the present cohort was only constituted of survivors and that there was no patient in a vegetative state, my categorization of recovery as excellent versus incomplete seemed more useful, and provided a valid indication of patients' long-term clinical condition consistent with other measures of recovery: even though hospital and coma duration can be influenced by other factors than the cerebral insult itself (e.g. medical complications), they are overall accurate indicators of the time necessary to recover. In addition, the decision to refer patients to an intensive neurorehabilitation center accurately reflected global neurologic impairments in our cohort.

Time of assessment

In the present study, outcome was assessed at two time-points. Cognitive performances were evaluated shortly after awakening (on average 10 ± 7 days), while global functional outcome was characterized at 3 months. The early neuropsychological assessment allows identifying precociously patients suffering from significant cerebral damage, while reducing the influence of external factors to the brain injury (e.g. rehabilitative treatment, motivation, etc.), thus offering a direct relationship between acute brain processing and cognitive functions. Such bedside neuropsychological screening has been strongly associated with long-term cognitive performances (Prohl *et al.*, 2007, 2009), which is confirmed here with

worse global outcome in patients having severe cognitive deficits on the early assessment. These results support the predictive value of early neuropsychological assessment for long-term recovery. Our study goes even further by predicting both cognitive performances at bedside and long-term functional recovery based on EEG evaluations performed during coma.

3.2.2. The predictor: progression of auditory discrimination during coma

Auditory evoked potentials, and in particular the P300 and the MMN components, have been reliably related with survival in comatose patients (Daltrozzo *et al.*, 2007). However, these studies included patients from various coma etiologies (e.g. traumatic brain injury, stroke, anoxia), assessed AEPs at variables delays after coma onset (up to 96 days in (Naccache *et al.*, 2005)) and only very few of them evaluated the progression of such evoked responses (Wijnen *et al.*, 2007). In comparison, the present study, focusing on post-anoxic coma and characterizing the evolution of brain responses very acutely and systematically at the same latency after coma onset, should provide an unbiased representation of the comatose survivors' preserved functions and their recovery.

MMN paradigm

The mismatch negativity component is elicited by the detection of a rare and new event differing in some features from the frequent and similar stimulus repeatedly presented. Auditory MMN paradigms most often use one type of deviant sound (e.g. duration deviant: sound differing in duration as compared to the standard sound) to elicit this component. However, it has been demonstrated that different types of deviants might indicate different information in patients with schizophrenia (Todd *et al.*, 2008). In order to test whether such susceptibility also exists in comatose patients, our team built a MMN paradigm with three deviants (i.e. duration, frequency and localization deviants), and showed that the duration deviant was the best predictor of outcome among the three; however, even better results were obtained when combining all three deviants (Tzovara *et al.*, 2013). This is why I used the same MMN paradigm here and decided to average the decoding values of all deviants.

The method

As compared with previous studies assessing the MMN component with ERPs analyses at the level of single electrodes, the multivariate single-trial decoding algorithm used here has several advantages (Tzovara, Murray, Plomp, *et al.*, 2012). In particular, it selects on a data-driven way the most prototypical features characterizing the signal for each patient and each recording, separately. This allows an optimal decoding performance in each case, irrespective of patients' clinical conditions and brain injury potentially influencing signal topographies. It also circumvents the supposed similarity between healthy participants and

patients and from a priori hypotheses about the occurrence and the modulation of specific components (e.g. N100), classically used as inclusion criteria for further analysis (e.g. MMN). Moreover, in conditions with high inter-trial variability such as in coma, such complex algorithm estimating a high number of parameters may better capture this variability as compared to classical analyses (De Lucia and Tzovara, 2014).
In comparison to single electrode ERPs analyses, multivariate algorithm provides coherent results, for example concerning significant time periods, but also complementary information as specific voltage topographies reflect distinct underlying generators (Tzovara, Murray, Plomp, *et al.*, 2012).

3.2.3. How can the evolution of auditory discrimination predict the recovery?

The mismatch negativity component has been previously associated with cognitive and functional decline in healthy and clinical populations (Näätänen et al., 2011; Näätänen, Sussman, Salisbury, & Shafer, 2014). In particular, alterations of the MMN component have been associated with impaired cognitive functioning (Baldeweg *et al.*, 2004; Kärgel *et al.*, 2014; Kiang *et al.*, 2007) and strong evidences link MMN alterations to functional measures in patients with schizophrenia (Kaur *et al.*, 2013; Lee *et al.*, 2014; Light and Braff, 2005). In healthy controls, the MMN has shown significant correlation with psychosocial functioning but not with cognitive tests (Light *et al.*, 2007). Regarding verbal fluency, specifically, lower MMN amplitude and longer latencies have been associated with worse performances in patients with schizophrenia (Şevik *et al.*, 2011), while MMN amplitude predicted better verbal fluency scores in healthy boys and those with autistic spectrum disorders (Weismüller *et al.*, 2015). Interestingly, verbal fluency scores were the only neuropsychological measures exhibiting significant correlation with MMN amplitudes (Higuchi *et al.*, 2013), as opposed in particular to digit span performances (Higuchi *et al.*, 2013; Lin *et al.*, 2012). These results suggest that cerebral mechanisms involved in verbal fluency tasks might share specific features with processing of auditory regularities.
However, despite providing valuable insights, these findings can only be applied cautiously to the present results considering important divergences, concerning especially the clinical condition, the method used to characterize MMN and the fact that we describe the dynamics of auditory discrimination and not one isolated evaluation.

My results have some similarities with previous studies showing a significant relation between the long-latency somato-sensory evoked potential N70 and patients' outcome. The N70 peak is a cortical evoked potential, which is 100% specific of good outcome (CPC 1-2) (Madl *et al.*, 1993) but less reliable for the prediction of poor outcome (Madl *et al.*, 1993; Zandbergen *et al.*, 2006). Recorded on the 4th day of coma, it has been associated specifically with executive performances at 6 months follow-up (Prohl *et al.*, 2007). One could

therefore postulate that both N70 and auditory discrimination reflect the extent of the cortical brain injury, which can subsequently be related with cognitive performances.

3.2.4. Limitations

Only a relatively limited number of patients could be included in the present study, caused by the low rate of survival and the fact that one third of survivors were transferred very early to secondary hospital centers. However, this latter group was similar to our cohort in terms of global outcome, suggesting that the included sample was representative of the whole population of cardiac arrest survivors.

Also, here outcome prediction was based on the progression of auditory discrimination, which is only visible in about half of survivors. A worsening of discrimination can be observed both in non-survivors and survivors, and is therefore unspecific. The present results allow identifying most patients with extremely good recovery, while patients with worse outcome cannot be distinguished from non-survivors based solely on the evolution of auditory discrimination during coma. However, if complementary clinical investigations can establish survival, results of the progression of auditory discrimination could be used to reliably predict detailed functional outcome and therefore precociously guide the treatments in this population with worse recovery.

3.2.5. Future perspectives

Considering that patients' prognosis is usually clear within 3-4 days after coma onset and that pharmacological conditions are extremely standardized for the first two days, the present setting is optimal for including a maximum of patients with homogenous clinical conditions. However, it would be interesting to assess auditory discrimination every day until patients die or wake up in order to characterize the progression over a larger period of time. Indeed, it is possible that actually all survivors improve EEG brain responses at some point but that some (i.e. the "other survivors") exhibit this progression later, which could not be attested in the present setting. Such experimental design would bring more insight about how auditory discrimination evolves over time according to the subsequent functional outcome. Pursuing EEG recordings beyond awakening would also provide useful information regarding the difference of cerebral processing during and after the comatose state and has the advantage that each patient is his own control.

Considering that neuropsychological bedside screening in the first month after cardiac arrest seems to represent the best predictor of cognitive outcome at 6 months (Prohl *et al.*, 2009), the present results provide useful information for the prediction of long-term recovery. Further studies should confirm and extend these results by including such long-term assessment.

3.3. Trace conditioning during coma

In this third study, my goal was to investigate whether learning with trace conditioning can occur in the absence of consciousness, challenging current opinion that explicit awareness of the stimulus contingency is required. To this aim, I built a trace conditioning paradigm taking advantage of the EEG activity to investigate the creation of new associations at the neural level in deeply comatose patients. I evaluated the association by testing whether the neural activity elicited by the alerting sound would be reactivated when the alerting sound does not occur after the tone. Among the 29 patients included, eight of them (28%) showed such a reactivation effect, suggesting the creation of an appropriate expectancy of the occurrence of the alerting sound after the tone. In addition, when testing this reactivation effect at different adjacent latencies, I showed that the expectation was temporally specific of the exact interval of occurrence of the alerting sound. **Therefore, my results suggest that the comatose brain is able to build an internal model of the specific stimuli at precise time-periods, supporting the notion that consciousness might not be absolutely necessary for this type of learning.** To the best of my knowledge, this is the first attempt to evaluate learning based on electrophysiological activity in acute comatose patients.

3.3.1. Relations with clinical outcome

Among the eight patients exhibiting neural correlates of learning, only four (50%) recovered awareness and survived at 3 months, suggesting that the ability to associate auditory stimuli during coma is not predictive of better outcome, under the limitation of the restricted number of significant patients. Considering that the evolution of brain responses during coma has been related to patients' outcome (Tzovara *et al.*, 2013, 2015, 2016), I investigated such association for the present dataset, but did not found similar results. Such discrepancy could be at least partially explained by differences in tasks demands, as studies using more complex paradigms, such as discrimination of semantic stimuli (Cossy *et al.*, 2014) were not either reliable prognosis predictors.

3.3.2. Mechanisms underlying stimulus expectancy and learning

The sequence implemented in the trace conditioning paradigm entails the repeated presentation of couples of sounds, thus possibly inducing an expectancy of the second sound upon the presentation of the first. Recent models propose that the creation of predictions are at the basis of perception, and constitute a general principle of brain function (Friston, 2005, 2010). In particular, predictive coding refers to the formulation of expectations by higher areas of the cortical hierarchy based on the flow of incoming stimuli collected by lower sensory cortices (Friston, 2005). Higher areas send their predictions to lower areas as top-down signals. On this basis, lower areas will build an expected pattern of activation that will be contrasted with the sensory input received. A prediction error will be sent to higher

areas if there is any mismatch between the predicted and the actual pattern (Friston, 2005; SanMiguel, Saupe, *et al.*, 2013).

According to this theory, the signal obtained during the silent period following the conditioned tone ("conditioned silence") could be interpreted as a mix between the expected pattern built by sensory areas and the prediction error received by higher structures. In particular, previous evidence of reemerging brain activity during the missing period when a sensory input is expected but omitted has received converging lines of evidence in healthy subjects both in EEG (Chouiter *et al.*, 2015; SanMiguel, Saupe, *et al.*, 2013) and hemodynamic studies (Den Ouden *et al.*, 2009). As revealed by these previous studies, the neural substrates of this type of learning most likely involve both sensory areas and higher associative areas.

3.3.3. Prediction of the "what" vs. prediction of the "when"

Following the same idea, predictive timing refers to temporal expectations based on temporal regularities or associative contingencies (Arnal and Giraud, 2012). Mechanisms of temporal prediction are different from mechanisms of identity prediction (SanMiguel, Saupe, *et al.*, 2013). Temporal expectations can be explicitly modulated by attention or implicitly triggered by temporal properties of stimulus sequence (Lange, 2009). Current models suggest that temporal prediction is a modulatory process generating ideal windows for stimulus processing (Large and Jones, 1999; Schroeder and Lakatos, 2009). The neural responsiveness of sensory areas is supposed to be increased at the predicted time-windows, privileging the processing of stimuli arriving at this precise moment (SanMiguel, Saupe, *et al.*, 2013; SanMiguel, Widmann, *et al.*, 2013). In the present study, this may explain why the reactivation effect is higher at the precise latency of the alerting sound than at adjacent time-windows.

3.3.4. Limitations

Considering the high arousing and potentially unpleasant value of the alerting stimuli, I carefully included only those patients with the lowest scores on all the clinical tests assessing consciousness. This criterion was necessary for testing learning in the absence of consciousness and for avoiding emotional reactions to highly arousing stimuli in partially awakening patients. The FOUR score of all included patients was around 2-3 points for both hypothermic and normothermic recordings, which represents a very low score, strongly suggestive of deep coma. On the other hand, misclassification have happened using some other clinical tests (i.e. coma recovery scale (Giacino *et al.*, 2004)) in chronic patients such as vegetative or minimally conscious (Bekinschtein, Dehaene, *et al.*, 2009; Monti *et al.*, 2010; Owen *et al.*, 2006). In this context, it cannot be excluded that the current state of the art of

consciousness assessment in acute coma might show similar limitations in future investigations, imposing a reinterpretation of the current results.
The consequence of selecting only deeply comatose patients resulted in an increased number of recordings taking place on the first day of coma, where sedation and temperature management procedures ensure a lowest consciousness state. Combined with previous knowledge showing that post-anoxic comatose patients (and especially those not surviving), can have higher auditory discrimination performance during the first day of coma and under hypothermia than during following days (Tzovara *et al.*, 2013, 2016), this selection bias in my recordings could have lead to artificially increase the number of patients showing the reactivation effect. A further open question is whether this higher auditory discrimination is related to the time from the initial insult or to the temperature, which could not be answered here considering the reduced number of significant recordings.

Concerning the rate of reactivation effect, I found only 9/43 significant recordings (21%) from 8/29 patients (28%), which can seem low but which is actually in accordance with other studies in disorders of consciousness. In addition, I used a very strict procedure to assess the reactivation effect where only patients having an auditory discrimination were included for further analysis, therefore importantly reducing the chances of finding significant results.

Finally, despite lower discrimination performance before and after the alerting sound period in all patients showing a significant reactivation effect, some recordings were also significant at these adjacent time-periods. This suggests that the temporal expectation of the alerting sound was not very precise in these patients. In the predictive timing framework, this may correspond to larger time-windows for the preparation of the upcoming stimuli processing, which might be an adaptive mechanism, considering the large inter-stimulus interval in the present experimental design.

3.3.5. Future perspectives

The experimental approach designed here to assess learning is innovative, and could be corroborated by confirmatory evidence using more classical approaches. In particular, it would be interesting to include physiological measures, such as modulation of heartbeat or skin conductance responses, which are often used to evaluate the anticipation of the unconditioned stimulus. In the same vein, one could also analyze modifications of the conditioned stimulus as a function of learning by comparing its evoked activity before and after it has been paired with the alerting sound. This would provide additional evidence that the conditioned stimulus acquired complementary information about the subsequent occurrence of the alerting sound. In addition, the same paradigm in healthy participants may

be run in order to obtain a normal performance, even though this would probably provide distinct results in comparison to patients with different degree of severity of clinical condition.

This study opens the field of the investigation of neural correlates of learning in non-responding individuals. Building on these initial results, further experiments should test to which extent learning during unconsciousness is limited to very relevant stimuli, based on their emotional or arousing value, or whether it can happen even for more abstract associations.

In addition, it remains unknown to which extent the reactivation effect depends on the temporal interval between the conditioned tone and the alerting sound. In the present work, I used a fixed temporal interval to increase the repetitive characteristic of the association, but a paradigm with changeable intervals would maybe trigger more flexible mechanisms of learning, which may require conscious awareness of the contingency.

Finally, extending this study to other etiologies may prove useful to corroborate the present findings.

4. GENERAL CONCLUSIONS

Taken together, my results suggest that both clinical and experimental EEG investigations provide meaningful information regarding the state of preservation of the brain in comatose conditions. In particular, I showed that the evolution of specific clinical EEG patterns, i.e. background EEG reactivity, is associated with different clinical evolution during coma (first study), while the improvement of the evoked activity in response to auditory deviant stimuli is associated with better functional outcome of the survivors (second study). In addition, the investigation of neural correlates of the association between two stimuli suggested that some comatose patients can exhibit signs of learning, irrespective of their subsequent outcome (third study).

For two out of these three studies, I recruited patients from a single hospital center. This limited the number of included patients but conversely allowed a satisfactory consistency of acquired measured and clinical management, as the same teams provided clinical care and complementary tests for all patients. Moreover, I included only post-anoxic comatose patients, and furthermore essentially those treated with TH, which represents a very specific condition. Extending these studies to patients with controlled normothermia or no temperature management, and to other coma etiologies such as traumatic brain injury or stroke, would be important to generalize the present results. Besides that, a major strength in my studies is that none of the EEG investigations performed during coma were used to decide the interruption of intensive care measures, which considerably limits the risks of a “self-fulfilling prophecy”.

The present results suggest that the dynamics of brain responses over time during coma is informative of patients’ clinical recovery. This concept is very new as compared to the classical single-time evaluation, and should be investigated in further studies using different stimuli, paradigms, times of recording, and clinical etiologies. In addition, complementing these experimental approaches with well-known clinical examinations provides interesting assumptions and valuable insights about the brain mechanisms involved, and should be more systematically performed. Finally, my results contribute to improve the understanding on the residual cognitive functions occurring in unconscious conditions, which could have significant impact for the management and treatment of patients with acute disorders of consciousness. Besides predicting prognosis and providing complementary information on brain preservation, the present findings could be used to inform clinicians and relatives of the extent of stimuli that the patient can process, which could open the field to basic communication.

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6. ARTICLES

6.1. Clinical evolution after a non-reactive hypothermic EEG following cardiac arrest

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ORIGINAL ARTICLE

Clinical Evolution After a Non-reactive Hypothermic EEG Following Cardiac Arrest

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Abstract
Background Lack of electroencephalography (EEG) background reactivity during therapeutic hypothermia (TH) has been associated with poor outcome in post-anoxic comatose patients. However, decision on intensive care withdrawal is based on normothermic (NT) evaluations. This study aims at exploring whether patients showing recovery of EEG reactivity in NT after a non-reactive EEG in TH differ from those remaining non-reactive.
Methods Patients with non-reactive EEG during TH were identified from our prospective registry of consecutive comatose adults admitted after successful resuscitation from CA between April 2009 and June 2014. Variables including neurological examination, serum neuron-specific enolase (NSE), procalcitonin, and EEG features were compared regarding impact on functional outcome at 3 months.
Results Seventy-two of 197 patients (37 %) had a non-reactive EEG background during TH with thirteen (18 %) evolving towards reactivity in NT. Compared to those remaining non-reactive ($n = 59$), they showed significantly better recovery of brainstem reflexes ($p < 0.001$), better motor responses ($p < 0.001$), transitory consciousness improvement ($p = 0.008$), and a tendency toward lower NSE ($p = 0.067$). One patient recovering EEG reactivity survived with good functional outcome at 3 months.
Conclusions Recovery of EEG reactivity from TH to NT seems to distinguish two patients' subgroups regarding early neurological assessment and transitory consciousness improvement, corroborating the role of EEG in providing information about cerebral functions. Understanding these dynamic changes encourages maintenance of intensive support in selected patients even after a non-reactive EEG background in TH, as a small subgroup may indeed recover with good functional outcome.

Keywords EEG reactivity · Hypothermia · NSE · Post-anoxic coma · Prognostication · Anoxic-ischemic encephalopathy

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Introduction

Coma after cardiac arrest (CA) represents a severe condition with very uncertain outcome. In order to assist the clinician to perform early prediction on chances of survival and help in the decision of maintaining intensive life support, multimodal evaluations including brainstem reflexes. Motor response to painful stimuli, early myoclonus, electroencephalography (EEG), somatosensory evoked potentials (SSEP), and serum biomarkers [especially

neuron-specific enolase (NSE)] are part of current recommendations [1–5]. Furthermore, serum procalcitonin (PCT) has been recently described as a marker of post-resuscitation illness correlating with final outcome [6].

In the last decade, therapeutic hypothermia (TH) has been increasingly used in this clinical setting [7–9], although the exact parameters of temperature management have been recently challenged [10]. While it is important to try to predict as soon as possible the patient's outcome, TH itself and sedation have been shown to impact on the prognostic assessment [11, 12]. Current guidelines taking into account TH treatment recommend to assess prognosis multimodally at more than 72 h after CA onset, once the patient is back in normothermia (NT) and off sedation [13, 14].

Although clinical evaluation may be delayed with TH treatment, EEG is widely available, non-invasive, and seems to represent a relatively robust predictor, especially if recorded after at least 9–12 h following CA, particularly in terms of background reactivity [15–17]. Recently, we showed that despite a reactive EEG in TH, a discontinuous EEG and high NSE [but not serum procalcitonin (PCT)] correlate with mortality [18].

In order to further explore the dynamic role of EEG as a biological and prognostic marker in this clinical setting, we assessed the evolution of EEG features and clinical outcome in patients lacking background reactivity in TH. We hypothesized that return of EEG reactivity over time would define a subgroup of patients with a different clinical profile.

Methods

Patients and Procedures

We considered all patients from our prospective registry of consecutive comatose adults admitted between April 2009 and June 2014 to the Department of Intensive Care Medicine after successful resuscitation from CA. We included patients with both EEG recordings during TH and NT, selecting only those with a non-reactive EEG background in TH. All subjects were treated using a standardized protocol in agreement with current guidelines [9, 15] with mild TH to 33 °C maintained for 24 h; midazolam (0.1 mg/kg/h) and fentanyl (1.5 µg/kg/h) were given for sedation-analgesia, and vecuronium (0.1 mg/kg boluses) to control shivering. All data were collected prospectively. This study received full approval from the Ethic Commission of our hospital.

Clinical and Laboratory Variables

Neurological examination testing brainstem reflexes (pupillary, oculocephalic, corneal; all present vs. one or more absent), motor response to painful stimuli (flexion posturing or better vs. extension or no response) and myoclonus occurrence was performed repetitively after rewarming and up to 72 h after CA; the best evaluation was considered for this analysis. Serum NSE at 24 h and/or 48 h after CA was analyzed with an automated immunofluorescent assay (Thermo Scientific Brahms NSE Kryptor Immunoassay); the highest value was selected for this analysis. Serum PCT was sampled during TH using the ELFA method (Vidas Brahms PCT assay, bioMerieux Inc., Geneva, Switzerland; see [6]). Response to median-nerve SSEP was recorded 24–72 h after CA, after rewarming [15].

EEG Recordings

Video-EEGs (Viasys Neurocare, Madison, WI, USA) were recorded for 20–30 min using a 21-electrodes montage according to the international 10–20 system. For each patient, two EEG recordings were performed, the first time early after coma onset, during TH (range 2–36 h after CA; temperature at 33–34 °C), the second time after rewarming over 35 °C and most often after sedation weaning (range 24–72 h after CA). EEG activity was visually interpreted, before knowing the clinical outcome, by one experienced EEG-certified neurologist (AOR or JN) on three dimensions (see [18] for details): (1) background reactivity, categorized as present if clear and reproducible change in amplitude or frequency in the background occurred in reaction to stimulations, excluding muscle artifacts or stimulus-induced rhythmic, periodic, or ictal discharges (SIRPIDs); (2) spontaneous discontinuous pattern, defined as an interruption of the EEG background by flat periods during >10 % of the recording [19]; (3) epileptiform activity. In case of doubt about the qualification of the signal, the second EEG-certified neurologist was consulted and a consensus found.

Decisions on Intensive Care Withdrawal and Outcome Assessment

Intensive care withdrawal was discussed interdisciplinary within 7 days of CA, and based on the occurrence of two or more of: (1) unreactive EEG background in normothermia, (2) incomplete recovery of brainstem reflexes, (3) early myoclonus resistant to treatment, (4) bilateral absence of N20 cortical potential in SSEP tested in normothermia [20]. Importantly, EEG evaluation in hypothermia, NSE, and PCT values were not considered for this decision. Outcome was assessed at 3 months by a semi-structured phone interview and categorized according to the Cerebral Performance Categories (CPC; [21]).

Statistical Analysis

Two-sided Fisher and Wilcoxon tests were used to explore relationships between patients recovering EEG reactivity after return to NT versus those remaining without reactivity. Significance was set at $p < 0.05$, without correction for multiple comparisons, given the exploratory character of this study. Calculations were performed with Stata software, version 12 (College Station, TX).

Results

During the study period, 246 patients were admitted for CA and treated with TH but 49 were excluded for lack of EEG evaluation during TH (Fig. 1). Among the resulting 197 patients, 72 (37 %) had a non-reactive EEG in TH, with 59 (82 % of them) remaining non-reactive during NT and 13 (18 %) turning to be reactive in NT. Overall, outcome was poor, as 71/72 patients died (CPC 5). Demographic and clinical variables are summarized in Table 1. Groups did not differ according to age, sex, etiology, and latency to the first EEG in TH. Significant differences between groups

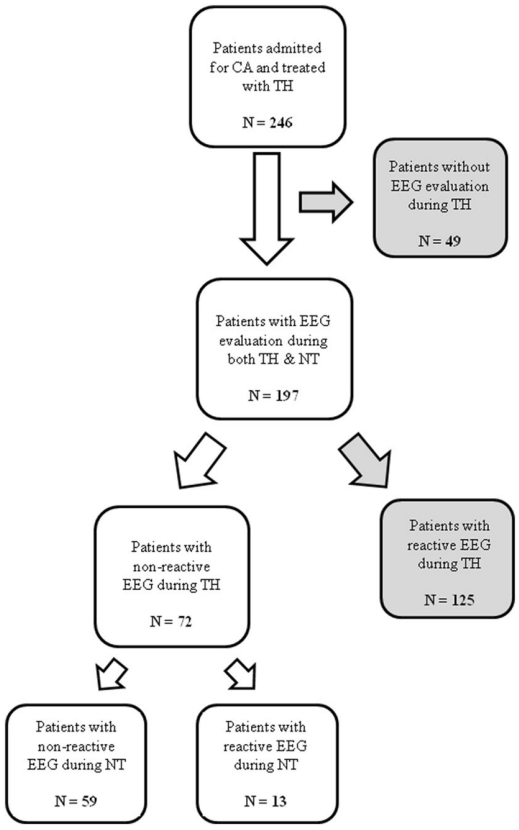

Fig. 1 Distribution of patients admitted after successful resuscitation from CA according to EEG background reactivity

with non-reactive EEG (NTnrEEG) versus reactive EEG (NTrEEG) in NT were found on the proportion of patients having absent brainstem reflexes ($p < 0.001$) and absent motor response ($p < 0.001$). Although not significant, there was a strong tendency concerning lower NSE values in the NTrEEG group ($p = 0.067$). The proportion of patients recovering partial consciousness (vegetative state or better) at least transiently was significantly higher in the NTrEEG group compared to the NTnrEEG group ($p < 0.01$).

Latency between cardiac arrest and death was longer in the NTrEEG group ($p < 0.001$), but the proportion of patients having residual pharmacological sedation during EEG recording in NT was not significantly different between both groups. In order to assess whether sedation had an influence on these results, we repeated calculations without the four patients sedated in NTnrEEG group ($n = 55$) and the three sedated in the NTrEEG group ($n = 10$). Concerning brainstem reflexes, the difference was still significant (respectively, 87 % for NTnrEEG group and 30 % for NTrEEG group; $p < 0.001$), as well as for motor response (respectively, 98 and 60 %; $p = 0.001$), the best level of consciousness achieved (respectively, 4 and 30 %; $p = 0.02$), and death latency (respective medians [range] in days: 3 [1–12] and 7 [4–11]; $p < 0.001$).

The only survivor of this cohort without a reactive EEG in TH belonged to the NTrEEG group. This 80-year-old man had CA caused by ventricular fibrillation of a cardiac etiology, and 25 min to a return of spontaneous circulation (ROSC); his EEG in TH was non-reactive, discontinuous, without epileptiform transients, and became reactive but still discontinuous in NT; brainstem reflexes were all present, motor response was obtained and no myoclonus was observed; NSE peak value was 17.3 µg/l and PCT 0.06 µg/l. At 3 months, this man was considered having a good functional outcome (CPC 2: moderate disability).

Conclusions

This prospective study shows that patients with a reactive EEG in normothermia after a non-reactive hypothermic EEG tend to recover brainstem reflexes and better motor reactions to pain, than those remaining with a non-reactive recording. Outcome did not differ significantly in term of survival between the two groups; however, those with a reactive EEG background in NT had a greater chance to reach at least transiently a vegetative state, and one patient even awoke and survived at 3 months with good functional outcome.

Our results suggest that recovery of EEG background reactivity in NT parallels at least partial clinical recovery of cerebral functions. On the one side, motor response

Table 1 Clinical and electrophysiological data of 72 patients with non-reactive EEG during therapeutic hypothermia, stratified for EEG reactivity in normothermia

	Non-reactive EEG background in normothermia ($n = 59$)	Reactive EEG background in normothermia ($n = 13$)	p value	Test
Median age, years (range)	65 (27–86)	68 (20–88)	0.714	Wilcoxon
Female gender	17 (29 %)	2 (15 %)	0.491	Fisher
Cardiac etiology	36 (61 %)	11 (84 %)	0.196	Fisher
Median time to ROSC, min (range)	25 (5–75)	30 (15–83)	0.234	Wilcoxon
Pulseless electrical activity or asystole	36 (61 %)	5 (39 %)	0.215	Fisher
Median time to first EEG, h (range)	17 (2–36)	19 (5–31)	0.421	Wilcoxon
Discontinuous EEG in TH	56 (94.9 %)	12 (92 %)	0.558	Fisher
Epileptiform EEG in TH	30 (50.8 %)	4 (31 %)	0.231	Fisher
Median serum NSE peak, µg/l (range)	88.2 (9–1063) $n = 53$	54.6 (15–128)	0.067	Wilcoxon
Median serum PCT peak, µg/l (range)	1.43 (0.06–284.65) $n = 50$	1.03 (0.06–7.22)	0.289	Wilcoxon
Cortical SSEP bilaterally absent	34 (68 %) $n = 50$	4 (31 %)	0.124	Fisher
Discontinuous EEG in NT	46 (78 %)	9 (69 %)	0.490	Fisher
Epileptiform EEG in NT	29 (49 %)	7 (54 %)	1.000	Fisher
Residual pharmacological sedation during EEG recording in NT[a]	4 (7 %)	3 (23 %)	0.106	Fischer
Brainstem reflexes absent[b]	52 (88 %)	5 (39 %)	<0.001	Fisher
Early myoclonus present	24 (41 %)	4 (31 %)	0.754	Fisher
Motor response worse than flexion posturing	58 (98 %)	8 (62 %)	<0.001	Fisher
VS, MCS, or awake at any time	2 (4 %)	4 (31 %)	0.008	Fisher
Survival at 3 months	0 (0 %)	1 (8 %)	0.181	Fisher
Median death latency, days (range)	3 (1–12)	6.5 (4–49) $n = 12$	<0.001	Wilcoxon

ROSC return of spontaneous circulation, *EEG* electroencephalography, *NSE* neuron-specific enolase, *PCT* procalcitonin, *SSEP* somatosensory evoked potential, *TH* therapeutic hypothermia, *NT* normothermia *VS* vegetative state, *MCS* minimal conscious state

[a] Drugs used: propofol, clonazepam, midazolam

[b] At least one of: pupillary, corneal, oculocephalic

reflects the integrated function of brainstem and higher cerebral structures; in fact, the cut-off in the motor GCS scoring is extension posturing or worse, defining at best a decerebrate state [22, 23], while, on the other side, pupillary, oculocephalic, and corneal reflexes reveal activity of brainstem function. Since EEG signals arise from the cerebral cortex, and are more sensitive to cortico-cortical connections than SSEP [24], EEG reactivity may reflect the dynamic integration of the cortex with the underlying structures. Despite this, however, clinical improvement was limited and transitory in most cases, pointing to the fact that EEG reactivity during TH and under relatively standard conditions, including sedation, may inform more accurately than in NT on the general "tuning" resulting from neuronal injury [25].

Serum NSE values tended to be lower in the group becoming reactive in NT, suggesting that the extent of neuronal damage is somewhat lower in this group of patients [25, 26]. However, overall, the median values were higher as compared to the previous study focusing on patients with an initial reactive EEG background in TH [18], and clearly above the threshold of 33 µg/l [4], corroborating the hypothesis that the presently studied cohort (having a non-reactive EEG in TH) had a more severe brain damage from the beginning. PCT correlates with systemic post-resuscitation illness [6], but was not different here; again, it does not seem that EEG, at least in this clinical setting, mirrors the extent of systemic illness. SSEP, reflecting the integrity of thalamocortical projections using the N20 response, is an indicator of poor outcome [24]; however, knowing that its sensitivity is very low, the absence of difference in our setting is not surprising.

This study has some limitations: first, the number of patients included is relatively limited and only few belonged to the group with reactive EEG background in NT; however, these numbers reflect the analysis of a large cohort of 197 patients recorded consecutively over more than 5 years. Second, some few data were missing concerning PCT, NSE, and SSEP, but this reflects a common problem in observational studies related to availability of selected investigations during weekends and holidays. Third, this study was carried out in one single center,

possibly limiting generalization of our results; however, we believe that this allows a better internal validity. Fourth, the fact that description of EEG activity was performed by two different neurologists could be a source of bias; however, we recently [27] showed that correlation among readers for reactivity has a reasonable agreement, which may be especially true in this particular setting as JN was trained by AOR and the two collaborate closely since many years. Moreover, the choice to use standard EEG and not continuous EEG should not impact on the validity of results, as the added value of the latter method was reported mainly for a marginal higher detection of epileptic seizures, without any change in outcome [28, 29] or assessment of reactivity, particularly during normothermia [16]. Conversely, the prospective nature of data ascertainment and the multimodal approach, including not only clinical and electrophysiological parameters but also biological markers, as well as the assessment of functional outcome at 3 months, strengthen in our view the results. Finally, self-fulfilling prophecy is always a risk in this type of studies, and as decision of intensive care withdrawal was based especially on reactive EEG background during NT, it seems logical that longer death latencies were found in the group converting to a reactive EEG background.

To our knowledge, this study represents the first attempt to better characterize the clinical profile of post-anoxic comatose patients having an initial poor electrophysiological assessment (non-reactive EEG in hypothermia). We show that although the initial clinical presentation of these patients seems homogenous, the evolution of EEG background reactivity in NT distinguishes two subgroups with different clinical profiles. By outlining differences between these two groups concerning both early clinical variables and outcome, our results encourage the maintenance of intensive life support in case of EEG background reactivity recovery in NT even after a non-reactive EEG background in TH, especially when this recovery appears along with improved clinical signs, as a few patients may indeed recover.

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Disclosures The authors declare that they have no other conflict of interest.

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6.2. Prediction of cognitive outcome based on the progression of auditory discrimination during coma

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Manuscript Draft

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Title: Prediction of cognitive outcome based on the progression of auditory discrimination during coma

Article Type: Original Article

Keywords: Post-anoxic coma, EEG, cognition, functional outcome, prediction

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Abstract: Aim: To date, no clinical test is able to predict cognitive and functional outcome of cardiac arrest survivors. Improvement of auditory discrimination in acute coma indicates survival with high specificity. Whether the degree of this improvement is indicative of recovery remains unknown. Here we investigated whether auditory progression over time can predict long-term cognitive and functional outcome.
Methods: We prospectively recorded electroencephalography responses to auditory stimuli of post-anoxic comatose patients on the first and second day after admission. For each recording, auditory discrimination was quantified, and its evolution over the two recordings was used to classify survivors as "predicted" when auditory discrimination increased vs. "other" if not. Cognitive functions were tested on awakening and functional outcome was assessed at 3 months using the Cerebral Performance Categories (CPC) scale.
Results: Thirty-two patients were included, 14 "predicted survivors" and 18 "other survivors". "Predicted survivors" exhibited a better cognitive status (ability to follow a standardized neuropsychological battery: 86% vs. 44%; p=0.03 (Fisher)) and were more likely to show an excellent functional outcome at 3 months (CPC 1: 86% vs. 33%; p=0.004 (Fisher)). Moreover, progression of auditory discrimination during coma was strongly correlated with cognitive performance on awakening (phonemic verbal fluency: rs=0.48; p=0.009 (Spearman)).
Conclusions: Progression of auditory discrimination during coma provides early indication of future recovery of cognitive functions. The degree of improvement is informative of the degree of functional impairment. If confirmed in a larger cohort, this test would be the first to predict detailed outcome at the single-patient level.

TITLE PAGE

Title: Prediction of cognitive outcome based on the progression of auditory discrimination during coma

Keywords: Post-anoxic coma, EEG, cognition, functional outcome, prediction

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The authors declare no conflit of interest.

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ABSTRACT

Aim: To date, no clinical test is able to predict cognitive and functional outcome of cardiac arrest survivors. Improvement of auditory discrimination in acute coma indicates survival with high specificity. Whether the degree of this improvement is indicative of recovery remains unknown. Here we investigated whether auditory progression over time can predict long-term cognitive and functional outcome.

Methods: We prospectively recorded electroencephalography responses to auditory stimuli of post-anoxic comatose patients on the first and second day after admission. For each recording, auditory discrimination was quantified, and its evolution over the two recordings was used to classify survivors as "predicted" when auditory discrimination increased vs. "other" if not. Cognitive functions were tested on awakening and functional outcome was assessed at 3 months using the Cerebral Performance Categories (CPC) scale.

Results: Thirty-two patients were included, 14 "predicted survivors" and 18 "other survivors". "Predicted survivors" exhibited a better cognitive status (ability to follow a standardized neuropsychological battery: 86% vs. 44%; p=0.03 (Fisher)) and were more likely to show an excellent functional outcome at 3 months (CPC 1: 86% vs. 33%; p=0.004 (Fisher)). Moreover, progression of auditory discrimination during coma was strongly correlated with cognitive performance on awakening (phonemic verbal fluency: r_s=0.48; p=0.009 (Spearman)).

Conclusions: Progression of auditory discrimination during coma provides early indication of future recovery of cognitive functions. The degree of improvement is informative of the degree of functional impairment. If confirmed in a larger cohort, this test would be the first to predict detailed outcome at the single-patient level.

INTRODUCTION

Cardiac arrest affects yearly more than 320'000 people in the US [1]. Half of the hospital admitted patients survive to discharge, mostly after post-anoxic coma, and among those about 50% may undergo some degree of long-term cognitive impairment [2,3]. In this condition, predicting survival, and especially cognitive and functional outcome, is a major concern for clinicians and relatives. To date, clinical evaluations during coma (such as clinical examination, neurophysiological tests, biochemical markers, and brain imaging) are used to predict poor outcome [4,5]. Previous studies investigating the contribution of these variables for the prediction of functional outcome are sparse and showed inconclusive results. Only one study reported that the S-100B protein (reflecting glial suffering) correlated to long-term cognitive performances [6], but these results have never been replicated.

In contrast to these clinical evaluations, assessment of auditory functions during coma showed promising results in predicting survival [7–1112]. Patients' auditory response is typically quantified by measuring the so-called mismatch negativity (MMN) component, elicited automatically upon occurrence of a deviant stimulus in a train of regularly repeated stimuli [13]. The presence of an MMN in comatose patients from various etiologies has been correlated with awakening from coma [14–16]. Using a multivariate EEG decoding algorithm, our group previously demonstrated that an improvement in auditory discrimination between the first and the second day of coma predicted survival at 3 months in patients treated with therapeutic hypothermia [10,11]. However, some survivors did not show any improvement in decoding performance. This raises two questions about the contribution of auditory discrimination to outcome prediction: (1) Is this difference informative of survivors' functional outcome? (2) Is the degree of improvement indicative of the degree of recovery?

In this study, we measured the evolution of auditory discrimination during acute coma and assessed functional outcome at awakening in a cohort of cardiac arrest survivors, hypothesizing that the progression of auditory discrimination during early coma mirrors brain recovery measured by neuropsychological and functional evaluations. More specifically, we expected that survivors showing improved auditory discrimination during coma would exhibit better cognitive and functional outcome, and postulated an association between the progression of auditory discrimination and outcome measures. To our knowledge, this is the first attempt to use the evolution of auditory processing during early coma for prediction functional status in this clinical setting.

METHODS

Study design and population

Between October 2012 and September 2015, 119 adult post-anoxic comatose patients admitted to the Department of Intensive Care Medicine of the Lausanne University Hospital (CHUV). Ninety-six (81%) could be prospectively recorded with EEG on the first and second day of coma using the MMN paradigm. About half of them (49; 51% of tested patients) awoke from coma and and 17 (37%) could not be tested neuropsychologically shortly after awakening, due to early transfer to other hospitals. Therefore, the analysed cohort is constituted of 32 patients (9 women; mean age ± standard deviation: 56 ± 14 years).

All but five patients were treated with mild therapeutic hypothermia to 33-34°C for the first 24 hours, induced through ice packs and the Arctic Sun® system surface cooling device (Medivance, Louisville, CO, USA), in agreement with current guidelines [17]. Midazolam (0.1 mg/kg/h) and fentanyl (1.5 µg/kg/h) were given for sedation-analgesia, and vecuronium (0.1 mg/kg boluses) in case of shivering. Patients with myoclonus or electrographic epileptic seizures received intravenous, nonsedating anti-epileptic treatment (valproate, levetiracetam). Return to normal temperature was controlled at 0.5°C increase per hour until 37°C. Sedation was discontinued at 36°C. Four out of the five patients not receiving therapeutic hypotherma had targeted temperature management at 36°C for 24h, the remaining patient had no temperature control; all were sedated as the hypothermic patients. The decision to prescribe hypothermia was taken according to patient's clinical situation and following latest recommendations [18]. On the second day, all patients were normothermic.

Decision to withdraw intensive care was discussed interdisciplinary within 7 days after admission, based on a multimodal approach [19]. Extubation was decided based on the clinical status (oriented and reproducible motor response to commands), in absence of respiratory problems. Importantly, auditory discrimination was not used to take any of these decisions, and its results were not communicated to the healthcare teams.

This study received full approval from our Ethic Commission. Informed consent was obtained in the first days from a family member or a physician not involved in the research protocol. After awakening, agreement was asked again directly to the patient or to a close relative.

Mismatch Negativity paradigm

As described in details in a previous study [10], we used an auditory MMN paradigm including one standard and three types of deviant sounds (pitch, duration and location deviants). Stimuli were separated by a constant interval of 950 ms between the onsets of two sounds. Standards were 1000 Hz sinusoidal tones of 100 ms duration and 0 ms inter-aural time difference. Pitch deviants were at 1200 Hz, duration deviants lasted 150 ms, and location deviants had 700 ms inter-aural time difference (left ear leading). Standard sounds were presented in 70%, while each type of deviant was presented in 10% of trials. A sequence included 500 standard sounds and 50 of each type of deviant sounds organized in a pseudo-random order. The same sequence was used in three consecutive blocks. Auditory stimuli were displayed at 90dB via specialized ER4 Etymotic earphones (Etymotic Research, Inc.) using E-prime 2.0 software for patients included up to 2014 (Psychology Software Tools Inc., Pittsburgh, PA) or the Psychophysics Toolbox (Psychtoolbox-3) extensions in Matlab afterwards [20–22]. The procedure was identical for recordings on the first and second day of coma.

EEG acquisition and pre-processing

EEG recordings were performed as detailed previously [19,23] using a 19 electrodes montage following the international 10-20 system (Viasys Neurocare, Madison, WI, USA; sampling rate of 1024 Hz, online reference to Fpz). For each patient, two recordings were collected: on the first day after admission, during hypothermia or normothermia but always under active sedation, and on the second day of coma, during normothermic conditions (at least 35°C), after sedation weaning. The recording included the MMN paradigm, run after the routine clinical part lasting 20-30 minutes [10,11].

Preprocessing was performed using Cartool v.3.43 [24]. We extracted peri-stimulus epochs spanning 50 ms before to 500 ms post-stimulus onset. An artefact rejection criterion of ±100 µV was applied offline. Data were re-referenced offline to the common average reference, 0.18–40 Hz band-pass filtered, and 50 Hz notch filtered. No prestimulus baseline correction was applied.

Decoding of single-trial EEG

As in our previous studies, we analysed auditory evoked responses to standard and deviant sounds using a multivariate EEG analysis [25,26], extracting in a data-driven manner time periods and prototypical voltage topographies discriminating between the two conditions. This offers the advantage of being free of *a priori* hypotheses about electrode location where a stimulus-related activity would be expected, and is independent of any inclusion criteria aside from having sufficient artifact-free trials. Because this method is based on voltage topographies, an accurate performance results from activation of different underlying neural generators between experimental conditions [26].

We applied analyses separately for each patient and recording to discriminate the neural responses to standard versus deviants. Artifact-free trials were divided in a training dataset, used to model the distribution of voltage topographies by a Mixture of Gaussians, and a test dataset that applied the resulting model to classify each single-trial. The decoding performance was measured as the area under the receiver operating characteristic curve (AUC) [27], quantifying the difference in brain responses to standard versus deviants from 0 to 1 (1 = perfect decoding), and averaged across the three types of deviant sounds, to obtain a unique value for each recording. Progression of auditory discrimination was calculated as the difference between the first and the second recording.

Early cognitive functioning

Few days after awakening (defined as the extubation), after and in a standard inpatient unit, cognitive functioning was tested by neuropsychologists blinded to auditory discrimination results using a standardized neuropsychological battery requiring 90 minutes. It included validated tests of language, praxia, gnosia, long-term and short-term memory, executive functions, and attention (see Supplementary material for a detailed description). .

Functional outcome

Long-term global outcome was assessed using the Cerebral Performance Categories scale (CPC; Booth, Boone, Tomlinson, & Detsky, 2004) at 3 months follow-up categorized as “excellent” for CPC 1 and “moderate” for CPC 2-3; No patient was vegetative (CPC 4). CPC scores were collected through a short semi-structured phone interview by a research nurse blinded both to auditory discrimination and cognitive testing.

Coma duration (time from admission to extubation; reflecting rapidity of brain function recovery), hospital stay duration (time from admission to discharge from any acute unit; reflecting recovery speed of global functions), and indication to neurorehabilitation were considered as additional variables of interest. Indication for intensive neurorehabilitative treatment reflects an interdisciplinary decision; since only patients with serious cognitive impairment were offered this option, this may be seen as a surrogate of the quality of recovery.

Relation between progression of auditory discrimination and outcome

We applied Spearman's rank correlations to investigate this relationship. In order to test whether "predicted survivors" and "other survivors" differed in their outcome, we compared cognitive and functional measures, using two-sided non-parametric tests (Wilcoxon signed rank test) or categorical tests (Fisher exact test), as appropriate. We did not correct for multiple comparisons given the exploratory nature of this study. All analyses were run on Matlab 2011b.

RESULTS

Auditory discrimination results during coma

Among the 32 tested survivors, 14 (44%) showed an increase in auditory discrimination over the first two coma days ("predicted survivors"), while 18 did not improve ("other survivors") (Fig. 1). The mean (± SEM) decoding performance for the first recording was 0.60 ± 0.009 for "predicted survivors" and 0.63 ± 0.006 for "other survivors", and 0.64 ± 0.009 and 0.59 ± 0.006 for the second recording, respectively. Nine of the 32 patients recovered minimal consciousness (still intubated but at times responding to commands) at the moment of the second recording (five in "predicted survivors", four in "other survivors").

Cognitive functioning on awakening

Neuropsychological testing took place on average 10 ± 7 days (here and in the following: mean ± standard deviation) after awakening. Fig. 2 shows detailed test results). More than one third of patients (12/32; 38%) could not perform the whole battery, and 14 (44%) were not oriented to time. Phonemic verbal fluency was impaired in 15/29 tested patients (52%), while this was the case in 11/28 (39%) for semantic verbal fluency and in 4/31 (13%) for digit span forward

Functional outcome

At 3 months follow-up, 18 (56%) patients showed excellent (CPC 1) and 13 (41%) moderate recovery (ten with CPC 2, three with CPC 3); one died few weeks after awakening (CPC 5) after decision to provide palliative care in a poor prognosis context related to severe comorbidities. The average coma duration was 6 ± 5 days, and hospital stay was 22 ± 18 days; 12/32 (38%) patients were addressed to a specialized neurorehabilitation center. Among the 17 subjects early

neuropsychological examination, nine (53%) displayed “excellent” outcome, seven (41%) “moderate”, and one died after awakening ((p = 1 as compared to tested patients, Fisher).

Relation between progression of auditory discrimination and outcome

We included in this analysis only cognitive measures available for most patients, i.e. ability to complete the whole battery, phonemic and semantic fluency, digit span forward, and orientation to time. Auditory discrimination progression during early coma and cognitive outcome measures showed significant positive correlations for both phonemic (r_s = 0.48, p = 0.009) and semantic verbal fluency scores (r_s = 0.45, p = 0.02), indicating that higher auditory discrimination improvement was associated with better cognitive performance (see Fig. 3A and Fig. 3B respectively). Moreover, progression of auditory discrimination showed a negative relationship with both coma duration (r_s = -0.4, p = 0.02) and hospital stay duration (r_s = -0.49, p = 0.005), meaning that higher auditory discrimination improvement predicted shorter recovery time (Fig. 4A and Fig. 4B respectively). These correlations survived when excluding possible outliers (coma duration: r_s = -0.4, p = 0.03; hospital stay duration: r_s = -0.49, p = 0.006). No such significant relationship was found for digit span forward scores.

Table 1 compares “predicted survivors” to “other survivors” on clinical variables, cognitive performances and functional outcome. Clinical characteristics did not differ between groups with respect to age, gender and hypothermia treatment. However, “predicted survivors” showed a tendency towards shorter cardiac arrest duration. Cognitive testing confirmed the above-mentioned correlations for phonemic and semantic verbal fluency, with higher scores for “predicted survivors”. In addition, more patients in the “other survivors” group were not oriented

to time and not able to perform the whole battery. Interestingly, time to cognitive assessment tended to be longer in the "other survivors" group, suggesting a slower recovery to a stable condition.

At 3 months follow-up, more "predicted survivors" exhibited a very good recovery (Table 1: CPC 1). Finally, "other survivors" showed both longer coma and hospital stay duration and were more likely to need a specialized neurorehabilitation center.

DISCUSSION

Our results show that the dynamic of auditory processing during early coma in cardiac arrest survivors provides accurate information about detailed functional status after awakening state. The evolution of auditory discrimination over the first two days seems to predict cognitive performances and rapidity of recovery. Grouping patients based on their progression of auditory discrimination yielded valuable information "predicted survivors" exhibited better cognitive functioning on awakening, faster recovery, and excellent functional outcome at 3 months (CPC 1), suggesting that the degree of progression of auditory discrimination could reflect a more general recovery of brain functions.

In comparison to the survival prediction, estimation of cognitive and functional outcome has received far less attention and is even more challenging, as most of clinical outcome predictors available during coma are predictive of death [4]. In contrast, auditory responses to mismatch negativity paradigms are more sensitive to survival [9,14], but have not been used to predict functional recovery despite encouraging results in healthy and psychiatric populations [29,30]. Compared to previous studies assessing auditory functions through mismatch negativity paradigms [8,9], here we took advantage of the whole EEG electrode montage to characterize the evoked brain activity. Its evolution over time has previously been reliably related with survival [10,11], but not with cognitive and functional outcome.

We characterized cognitive functioning using few measures available for the majority of patients according to their limited cognitive capacities shortly after awakening. In this context, ability to undergo the whole neuropsychological testing and orientation to time were interesting variables,

reflecting a generally preserved cognitive state. Verbal fluency represents a complex measure covering multiple cognitive domains, including primarily executive functions (e.g. self initiation, switching, inhibition), and verbal abilities [31]. Semantic and phonemic verbal fluency differ regarding both the involved neuroanatomical substrates and the recruited cognitive processes, with more executive processes and frontal involvement in phonemic verbal fluency [32,33]. Combined together, we believe that these measures may constitute a global and parsimonious appreciation of cognitive functioning. Of note, assessing cognition on awakening may reduce the influence of motivational and environmental factors (e.g. external stimulations, targeted neurorehabilitative treatments), highlighting more directly the relationship between acute cerebral processing and subsequent cognitive functioning.

We characterized global functional outcome at 3 months as CPC 1 vs. CPC 2-3, providing a discrimination between excellent and moderate recuperation. Even though coma and hospital stay durations can be influenced by many factors besides brain injury (e.g., infections or cardiovascular complications), they proved to be consistent with other measures and reliable indicators of the rapidity to recover. In particular, the tendency of "other survivors" to undergo neuropsychological testing later, while still having worse cognitive performance suggests, that longer time to recover is indicative of poor outcome. In addition, coma duration has been previously associated with functional status, showing more complaints of cognitive functioning and worse quality of life in patients awakening later [34,35]. Indication to intensive neurorehabilitative treatment was also a marker of poor outcome.

Interestingly, cardiac arrest duration tended to be shorter in "predicted survivors", suggesting that a briefer insult correlates to a better recovery of brain functions assessed by progression of auditory discrimination. In previous studies, cardiac arrest duration has been related to survival [36], but, as far as we are aware, never with detailed outcome[37,38], except in one older report showing a significant association with long-term memory scores [39]. The significant difference in arrest duration between the two groups of survivors, to the best of our knowledge our study is the first suggests that patients recovering auditory functions during coma (and later exhibiting better outcome) have lighter initial insult as attested by shorter time to ROSC.

The overall number of included patients is relatively small. Even considering all cardiac-arrest patients over three years in a university hospital, the very high mortality rate represents the highest constrain. However, functional outcome at three months of the few patients that could not be included in the present analysis did not differ significantly from those presented here, suggesting no selection bias. Therefore, we believe that our study represents a reliable sample of the population of cardiac arrest survivors. Some patients regained consciousness during the second EEG, but were almost equally distributed among the two groups; it seems thus unlikely that this influenced our results in a significant way. More importantly, results of auditory discrimination did not influence decisions on interruption of intensive care – minimizing risks of the so-called "self-fulfilling prophecy"; also, the neuropsychologist and nurse evaluating cognitive and functional outcome were blinded to them.

CONCLUSIONS

In summary, progression of auditory discrimination over time during early coma seems to reliably predict the subsequent cognitive and functional outcome of cardiac arrest survivors. Confirmation of these results for long-term outcome prediction in a larger cohort could contribute to more accurately orient early rehabilitation efforts and therefore improve patients' outcome.

Conflicts of interest

The authors declare no conflict of interest.

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Author contributions

Concept and study design: EJ, MDL, VB, MO, SC, AOR; Data acquisition and analysis: EJ, AT, VB, MDL; Drafting the manuscript and figures; EJ, MDL, AOR.

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FIGURE LEGENDS

Figure 1. Distribution of patients according to their progression of auditory discrimination during early coma, calculated as the difference of the decoding performance between the first and the second recording (respectively AUC_{Day1} and AUC_{Day2}). Patients showing an improvement of auditory discrimination are classified as "predicted survivors" while patients showing no improvement are categorized as "other survivors".

Figure 2. Number and percentage of patients impaired for each cognitive domain. Cognitive domains most frequently impaired were executive functions (53% of patients), long-term memory (35%) and attention (31%). Some cognitive domains could not be tested in all patients (praxia: n = 29, long-term memory: n = 31, short-term memory: n = 31).

Figure 3. Correlations between progression of auditory discrimination during early coma and cognitive scores on awakening. Positive correlations were found for both phonemic verbal fluency (panel A) and semantic verbal fluency (panel B).

Figure 4. Correlations between progression of auditory discrimination during early coma and functional outcome measures. Negative correlations were found both for coma duration (panel A) and hospital stay duration (panel B). Correlations remain significant without extreme values, indicated by empty rhombus (coma duration: r_s = -0.4, p = 0.03; hospital stay duration: r_s = -0.49, p = 0.006).

Table1

Table 1. Results of clinical variables, cognitive tests and outcome measures (mean ± std) for patients grouped according to their progression of auditory discrimination during acute coma.

	Predicted Survivors n = 14	**Other Survivors n = 18**	***p* value**	**Z value**	**Test**
Clinics					
Age (years)	57 ± 15	55 ± 15	0.82	0.22	Wilcoxon
Female gender	4 (29%)	5 (28%)	1		Fisher
Time to ROSC (min)	15 ± 6	24 ± 13	0.05	-1.97	Wilcoxon
Hypothermia treatment	12 (86%)	15 (83%)	1		Fisher
Cognitive functioning					
Cognitive testing delay (days)	8 ± 2	12 ± 9	0.07	-1.82	Wilcoxon
Whole NPS exam completed	12 (86%)	8 (44%)	0.03*		Fisher
Orientation to time failed	2 (14%)	12 (67%)	0.004*		Fisher
Phonemic verbal fluency (words)†	9 ± 4	4 ± 4	0.003*	2.95	Wilcoxon
Semantic verbal fluency (words)‡	16 ± 5	11 ± 7	0.02*	2.31	Wilcoxon
Digit span forward (span)¥	5 ± 1	5 ± 2	0.77	0.79	Wilcoxon
Functional outcome					
CPC 1 at 3 months	12 (86%)	6 (33%)	0.004*		Fisher
Coma duration (days)	4 ± 3	7 ± 5	0.01*	-2.51	Wilcoxon
Hospital stay duration (days)	15 ± 4	28 ± 22	0.003*	-2.93	Wilcoxon
Neurorehabilitation	1 (7%)	11 (61%)	0.003*		Fisher

ROSC return of spontaneous circulation, *CPC* Cerebral Performance Category

* Significant at $p < 0.05$

† Scores available for 29 patients (13 predicted survivors / 16 other survivors)

‡ Scores available for 28 patients (13 predicted survivors / 15 other survivors)

¥ Scores available for 31 patients (14 predicted survivors / 17 other survivors)

Figure1
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Figure 1

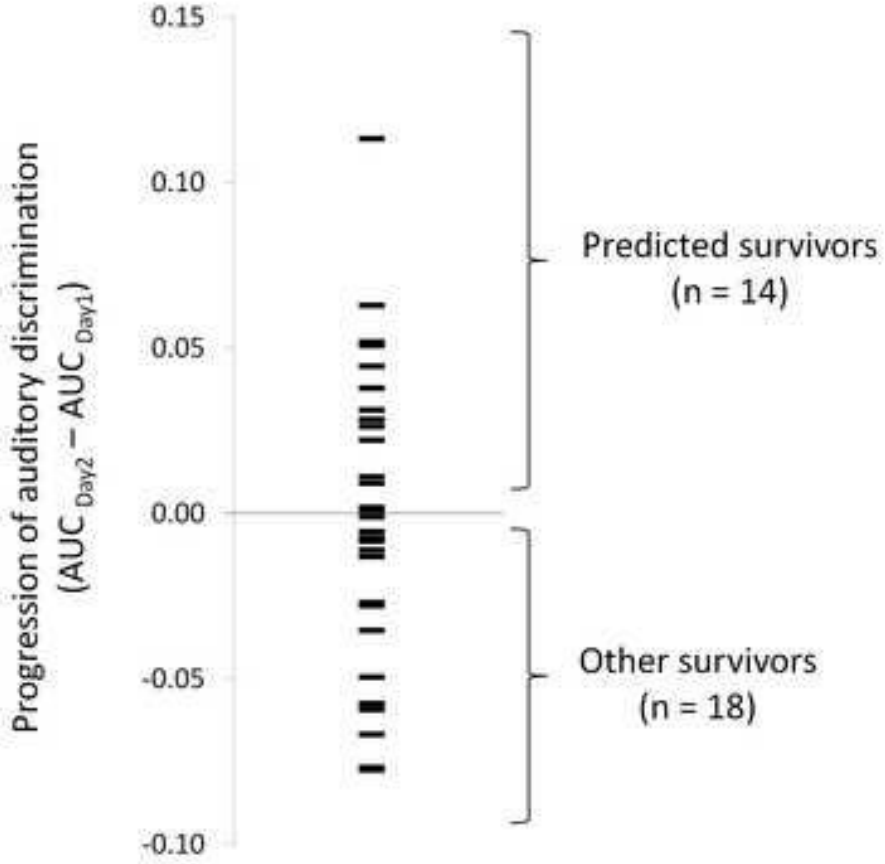

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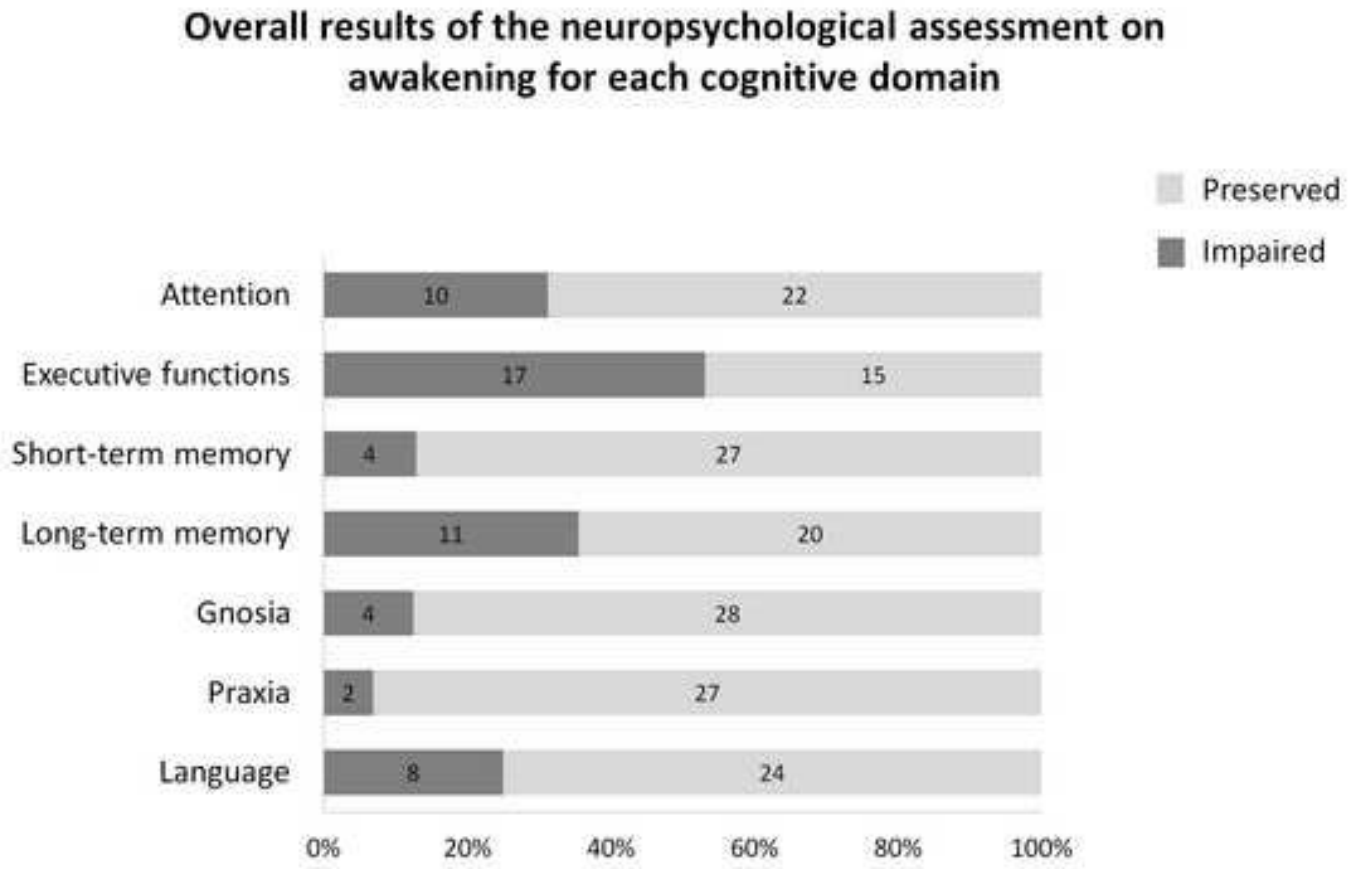
Figure 2



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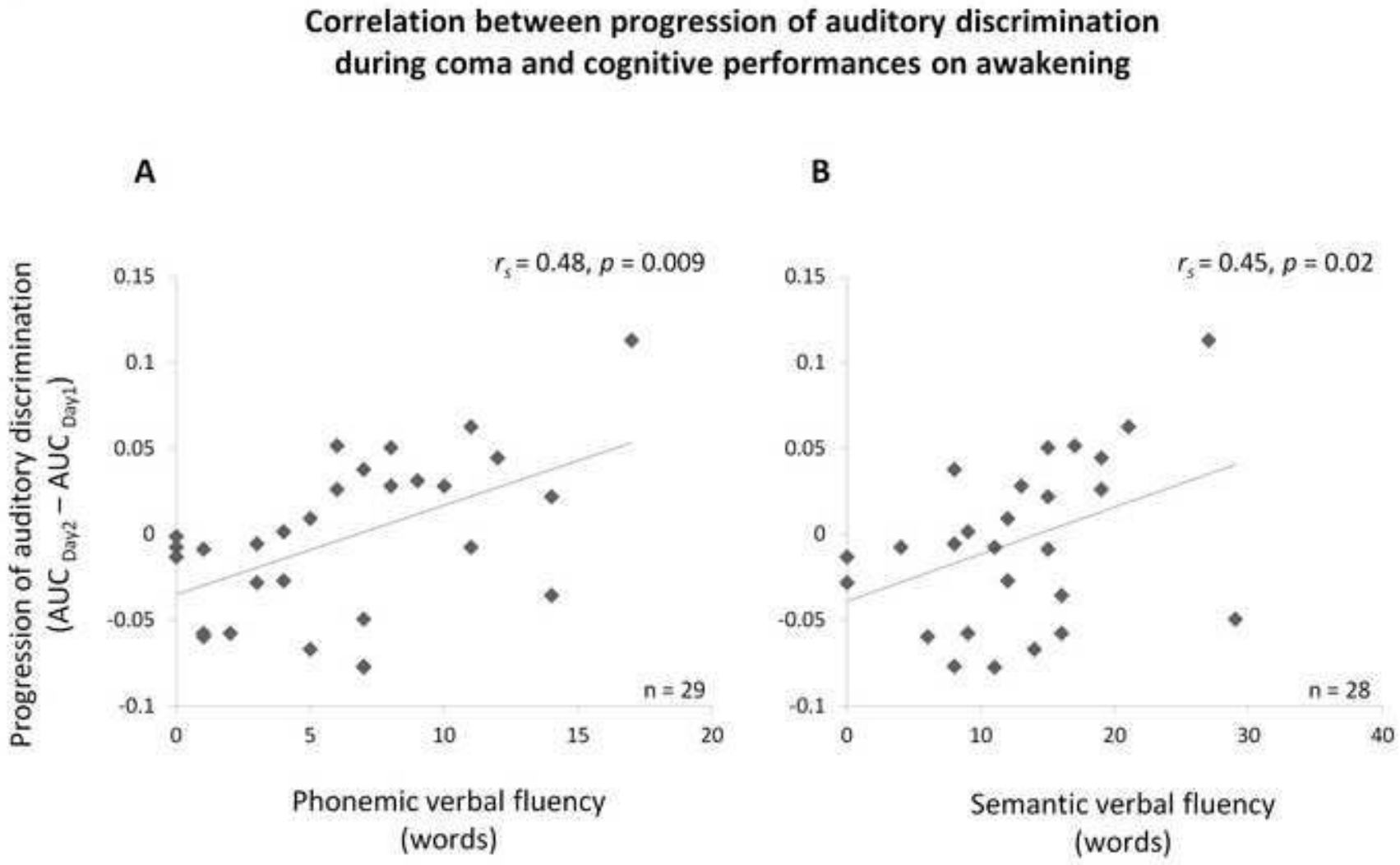

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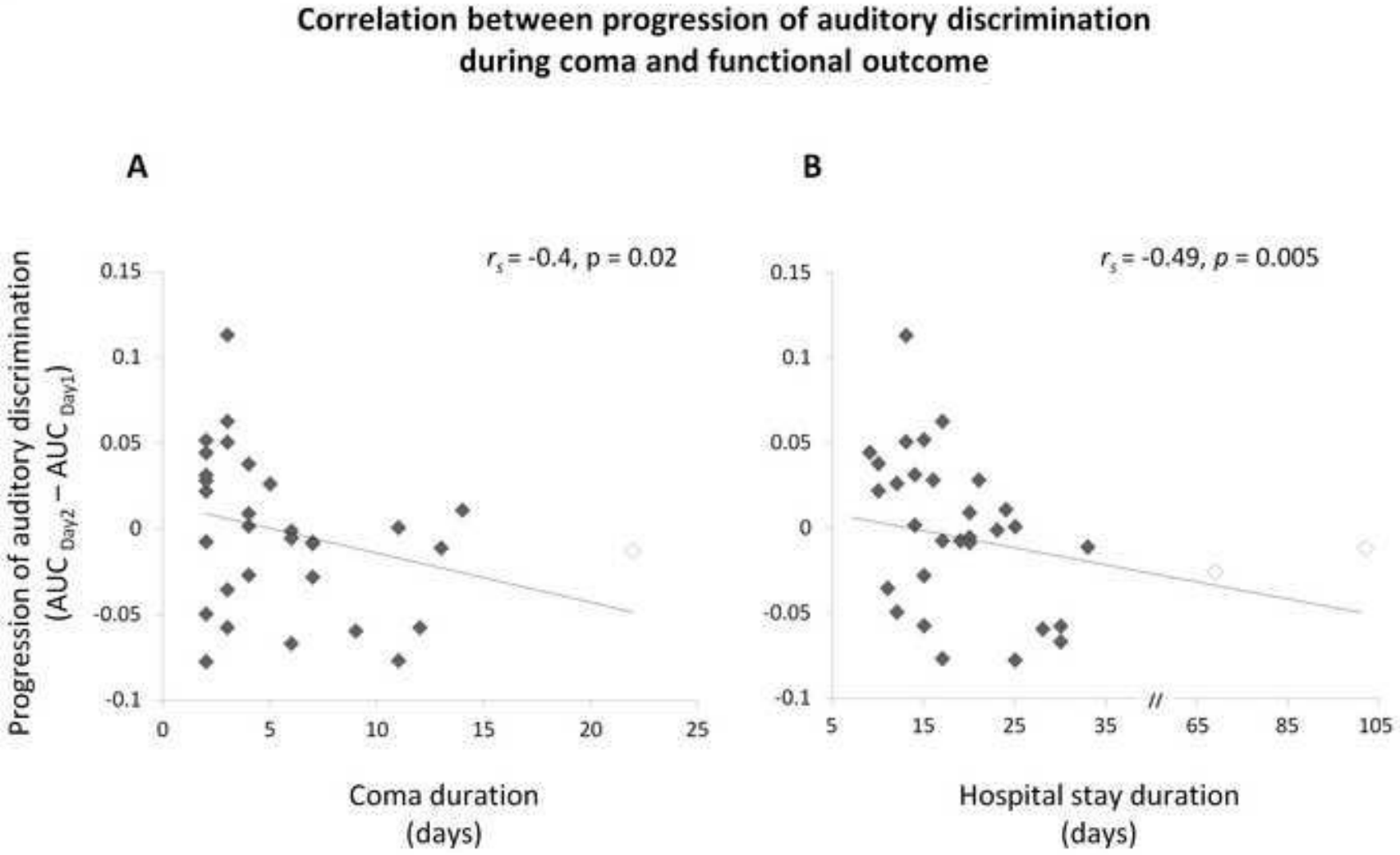

SUPPLEMENTARY MATERIAL

Description of the neuropsychological examination

The neuropsychological examination included a battery of tests covering the following cognitive domains:

(1) **Language**. Naming was tested with the French version of the Boston naming test (Thuillard Colombo & Assal, 1992). Other aspects of language were tested with a local protocol comprising repetition (tri-syllabic words and pseudo-words), oral comprehension (reversible, passive and apragmatic sentences) and written comprehension (reading and reporting a short text).

(2) **Visual gnosia**. Visuo-perceptive abilities were tested with the entangled figure recognition subtest of the Batterie d'Evaluation de la Négligence unilatérale (BEN; GEREN, 2002). Face recognition was evaluated with a local test assessing the identification of eight celebrities.

(2) **Praxia**. Constructive praxia were evaluated with the Praxis subtest of the CERAD battery (Welsh et al., 1994). Gestual praxia were tested with the Apraxia Screen of TULIA (AST; Vanbellingen et al., 2011).

(3) **Short-term memory**. Verbal short-term memory was evaluated using the digit span forward subtests of the French version of the Wechsler Adult Intelligence Scale, fourth edition (WAIS-IV; Wechsler, 2011). Visual short-term memory was assessed with the block tapping forward subtest of the French version of the Wechsler Memory Scale, third edition (MEM-III; Wechsler, 2001).

(4) **Long-term memory**. Verbal long-term memory was assessed with the 10 words (CHUV norms). Visual long-term memory was assessed with the 10 signs (CHUV norms).

(5) **Executive functions**. The Frontal Assessment Battery including concept elaboration (similitudes), generation (phonemic verbal fluency in 1 minute), environmental autonomy (grasping), programming (motor sequences), interference sensitivity (top/top-top) and inhibitor control (go/no-go) assessed global executive functioning (FAB; Dubois, Slachevsky, Litvan, & Pillon, 2000). Mental flexibility was evaluated with the Trail Making Test (Godefroy & GREFEX, 2008). Verbal inhibition was assessed using the color Stroop test (Godefroy & GREFEX, 2008). Non-verbal incitation was evaluated with the Five-points test (Goebel et al., 2009). In addition to phonemic verbal fluency, generation was also assessed with semantic verbal fluency (1 minute; CHUV norms).

(6) **Visual attention.** Visual search and selective attention were assessed with the Bell's subtest from the Batterie d'Evaluation de la Négligence unilatérale (BEN; GEREN, 2002). Space perception was assessed with the line bisection test (BEN; GEREN, 2002).

In addition to these tests, temporal, spatial and personal orientations, as well as writing and oral / written calculations were evaluated qualitatively.

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*Conflict of Interest Statement

Conflicts of interest

All authors disclose any financial and personal relationships with other people or organizations that could inappropriately influence the present work.

6.3. Evidence of trace conditioning in comatose patients revealed by the reactivation of EEG responses to alerting sounds

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Abstract: Trace conditioning refers to a learning process which occurs after repeated presentation of a neutral conditioned stimulus (CS+) and a salient unconditioned stimulus (UCS) separated by a temporal gap. Recent studies have reported that trace conditioning can occur in humans in reduced levels of consciousness by showing a transfer of the unconditioned autonomic response to the CS+ in healthy sleeping individuals and in vegetative state patients. However no previous studies have investigated the neural underpinning of trace conditioning in the absence of consciousness in humans.
In the present study, we recorded the EEG activity of 29 post-anoxic comatose patients while presenting a trace conditioning paradigm using neutral tones as CS+ and alerting sounds as UCS. Most patients received therapeutic hypothermia and all were deeply unconscious according to standardized clinical scales. After repeated presentation of the CS+ and UCS couple, learning was assessed by measuring the EEG activity during the period where the UCS is omitted after CS+ presentation. Specifically we assessed the 'reactivation' of the neural response to UCS omission by applying a decoding algorithm derived from the statistical model of the EEG activity in response to the UCS presentation.
We found a reactivation of the UCS response in absence of stimulation in eight patients, of which five were under therapeutic hypothermia. Additionally, the reactivation effect was temporally specific within trials since the reactivation manifested primarily at the specific latency of UCS presentation and significantly less before or after this period. Our results show for the first time that trace conditioning may manifest as a reactivation of the EEG activity related to the UCS and even in the absence of consciousness.

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TITLE PAGE

Title: Evidence of trace conditioning in comatose patients revealed by the reactivation of EEG responses to alerting sounds

Abbreviated title: Learning auditory associations during coma

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ABSTRACT

Trace conditioning refers to a learning process which occurs after repeated presentation of a neutral conditioned stimulus (CS+) and a salient unconditioned stimulus (UCS) separated by a temporal gap. Recent studies have reported that trace conditioning can occur in humans in reduced levels of consciousness by showing a transfer of the unconditioned autonomic response to the CS+ in healthy sleeping individuals and in vegetative state patients. However no previous studies have investigated the neural underpinning of trace conditioning in the absence of consciousness in humans.

In the present study, we recorded the EEG activity of 29 post-anoxic comatose patients while presenting a trace conditioning paradigm using neutral tones as CS+ and alerting sounds as UCS. Most patients received therapeutic hypothermia and all were deeply unconscious according to standardized clinical scales. After repeated presentation of the CS+ and UCS couple, learning was assessed by measuring the EEG activity during the period where the UCS is omitted after CS+ presentation. Specifically we assessed the 'reactivation' of the neural response to UCS omission by applying a decoding algorithm derived from the statistical model of the EEG activity in response to the UCS presentation.

We found a reactivation of the UCS response in absence of stimulation in eight patients, of which five were under therapeutic hypothermia. Additionally, the reactivation effect was temporally specific within trials since the reactivation manifested primarily at the specific latency of UCS presentation and significantly less before or after this period. Our results show for the first time that trace conditioning may manifest as a reactivation of the EEG activity related to the UCS and even in the absence of consciousness.

Significant statement

Learning the contingency between two previously unrelated sensory stimuli after their repeated presentation is an ubiquitous skill of the majority of animals. In humans, the occurrence of a temporal gap between stimuli is believed to make the conditioning highly dependent on the level of the subjects' perceptual awareness or consciousness level. Our study shows that trace conditioning can occur in a highly suppressed level of consciousness such as coma. Furthermore, we provide evidence, for the first time, of a reactivation of the neural response to the unconditioned stimulus, showing the specificity of the learned representation both in terms of sensory features and of its occurrence over time.

Keywords: learning, trace conditioning, coma, consciousness, EEG, multivariate analysis, auditory stimuli.

INTRODUCTION

Trace conditioning refers to the ability of learning the association between a neutral conditioned stimulus and a salient unconditioned stimulus when they are separated by a temporal gap. This learning phenomenon is of paramount importance for ensuring species survival, avoiding danger and optimizing reward (Bekinschtein et al. 2011).

The investigation around the minimal neural resources underlying trace conditioning in humans has led to controversial results. In particular, it remains elusive whether the awareness of the contingency between the conditioned and unconditioned stimulus is a necessary condition for such a learning phenomenon to occur and last over time. On one side, in awake healthy individuals the ability of the human brain of creating a trace of temporally separated sensory stimuli has shown a strong dependency upon subjects' awareness of the contingency (Carter et al. 2003; Christian & Thompson 2003). On the other hand, recent studies provided evidence that trace conditioning in humans can occur under reduced consciousness level: in some vegetative patients, repetitions of a sequence of neutral tones and air puffs triggered an anticipatory eye-blink response after the tone (Bekinschtein et al. 2009); a repeated presentation of neutral tones followed either by pleasant or unpleasant odors elicited a differential sniff response after the presentation of the sound alone in healthy individuals during deep sleep (Arzi et al. 2012). However consciousness level assessment remains uncertain in these previous studies: diagnosis of disorders of consciousness (i.e. vegetative or minimally conscious state patients) is affected by a considerable degree of misclassification (Godbolt et al. 2012; Schnakers et al. 2009), whereas consciousness level in healthy individuals during sleep as assessed by the global sleep scoring (Iber et al. 2007) is not sensitive to the presence of local wake activity (Dang-Vu et al. 2008; Le

Van Quyen et al. 2010), which might provide critical information on the consciousness depth during sleep (see discussion in Arzi et al. 2012). In addition, in anesthetized individuals, trace conditioning has not produced any significant result (Bekinschtein et al. 2009). Taken together, it remains open whether trace conditioning can be observed in the absence of consciousness. Furthermore, to the best of our knowledge, no studies have investigated the neural correlates of trace conditioning in altered state of consciousness (see also Kim et al. 2012; Lancioni et al. 2014 for learning assessment based on behavioral and autonomic responses).

Here we aim at testing the occurrence of trace conditioning based on comatose patients' EEG activity using an auditory protocol. Specifically, we used a differential trace conditioning paradigm with auditory stimuli presenting CS+ coupled with UCS on the majority of the trial and CS+ uncoupled on intermixed unexpected trials. Importantly the series comprised a control condition with a tone that was never associated to UCS, i.e. CS-. During the presentation of the paradigm, we recorded EEG activity of post-anoxic comatose patients. All of them were deeply unconscious based on standardized clinical tests and some of them under sedation and therapeutic hypothermia (TH) treatment (The hypothermia after cardiac arrest study group 2002; Bernard et al. 2002). We assessed trace conditioning by applying a single-trial decoding analysis testing the reactivation of the neural activity associated by the UCS when the UCS is expected but not presented after CS+ (Chouiter et al. 2015). Investigating the neural correlates of trace conditioning overcomes the difficulty of measuring autonomic responses to the conditioned stimulus in a deep unconscious state. Moreover, the use of EEG allows to assess the specificity of the association by comparing two different level of expectation states, i.e. after CS+ and after CS-. The high EEG temporal resolution provides insight about the temporal specificity of the reactivation effect within trial. Finally, by testing the occurrence of the reactivation phenomenon

along the duration of the experiment, we were able to unravel the appearance and persistence of the learning effect over time.

METHODS

Patients

We included data from 29 comatose patients in acute anoxic-ischemic coma (Glasgow Coma Scale < 6 at hospital admission; Teasdale & Jennett, 1974) following cardiac arrest. All patients were admitted to the Intensive Care Unit between March and October 2014 (5 women; mean age: 68 ± 2). Twenty-one were recruited from Lausanne University Hospital and 8 from Valais Hospital in Sion. Signed consent was obtained from patients' families according to a protocol approved by the Vaud and the Valais Ethics Committee.

Among these patients, 20 (2 women; mean age: 66 ± 2) were treated with a standard cooling procedure using therapeutic hypothermia at 33-34°C. At Lausanne University Hospital, this procedure is applied for the first 24 hours after coma onset through ice packs, intravenous ice-cold saline fluids and the Arctic Sun® system surface cooling device (Medivance, Louisville, CO, USA). Midazolam (0.1 mg/kg/h) and fentanyl (1.5 µg/kg/h) are given for sedation-analgesia and vecuronium (0.1 mg/kg boluses) in case of shivering. At Valais Hospital, hypothermia was maintained for 18 hours using the Coolgrad® intravascular cooling device (Zoll, Chelmsford, MA, USA), sedation was applied with disoprivan (2mg/kg/h) and fentanyl (50 µg/h); and norcuron (0.1 mg/kg boluses) was given in case of shivering. Patients suffering myoclonus or epileptic seizures received intravenous, nonsedating anti-epileptic treatment (valproate, levetiracetam). Return to normal temperature (normothermia; NT) after TH was controlled at a rate of 0.5°C increase per hour until 37°C and achieved within 8 hours. The remaining 9 patients (3 female; mean age: 73 ± 4) did not receive TH protocol for clinical reasons and were treated only with sedative drugs if needed.

Patients were recorded at one or several occasions in the course of their coma, depending on their clinical evolution and the EEG machine's availability. Hypothermic conditions occurred during the first day of coma only while normothermic conditions happened from the second day of coma in case of TH treatment or already from the first day of coma when TH treatment was not applied. A total of 43 recordings were performed, 19 during TH and 24 in normothermic conditions (7 recordings on the first day, 13 on the second day, and 4 on later days). Twelve patients were recorded twice and one patient was recorded three times. During recordings, all patients were intubated and with eyes closed. At the moment of the recording, body temperature was collected and consciousness was assessed using the FOUR score (Wijdicks et al. 2005).

Decision to withdraw intensive care was discussed within 7 days after coma onset, based on the occurrence of specific clinical variables strongly associated with poor outcome (Rossetti et al. 2010). Patients' outcome was assessed at 3 months in terms of survival (alive vs. deceased).

Stimuli

Two neutral tones of 100ms duration, 16-bit stereo 44100 Hz digitization were used as CS+ and CS- respectively at 700Hz and 900Hz frequency. A linear envelope of 5 ms increase time at the beginning of the tone and 5 ms decay time at the end of the tone was applied to minimize clicks. Tones were further normalized according to the root mean square of their amplitude.

As UCS, we used three alerting sounds selected on the ratings of healthy participants (see Supplementary material). Each of these three sounds was randomly chosen as UCS along the experiment to prevent the occurrence of an habituation.

Procedure and task

Our trace conditioning paradigm combined principles of differential conditioning by presenting two CS (CS+ and CS-) with a partial reinforcement procedure of CS+ presented in association with UCS (67% vs. 33% of CS+ alone occurrence). As displayed in Figure 1, three types of trials were presented randomly. Reinforced trials refer to the presentation of the CS+ followed by the UCS after a fixed inter-stimulus interval (ISI) of 800 ms (Figure 1, panel A). Non-reinforced trials designate the presentation of the CS+ without UCS; in these trials, the CS+ is followed by a silence of the same duration as the UCS (conditioned silence) after the same fixed ISI as in reinforced trials (Figure 1, panel B). Unpaired trials present the CS- alone and followed by a silence of the same duration as the UCS (neutral silence) after the same fixed ISI (Figure 1, panel C). Inter-trial interval (ITI) varies randomly between 1 and 2 seconds with steps of 0.1 seconds.

The same sequence was used in four conditioning blocks with ten additional reinforced trials presented consecutively at the beginning of the first block in order to create the association. The trial order was the same for all participants.

A total of 206 reinforced trials, 100 non-reinforced trials and 296 unpaired trials were presented, such as to have a similar number of CS+ (in reinforced and non-reinforced trials) and CS- presentation (unpaired trials). Each block lasted approximately 8.2 min for a total experiment time of 33 minutes.

Auditory sequences were presented at 90 dB using specialized ER4 Etymotic earphones (Etymotic Research, Inc.) while recording 19 channels EEG.

EEG acquisition and pre-processing

EEG recordings were performed using a 19 electrodes montage following the international 10-20 system (Viasys Neurocare, Madison, WI, USA) with a sampling rate of 1024 Hz and an online reference to the Fpz electrode.

Peri-stimulus epochs were extracted for the duration of the UCS, conditioned silence and neutral silence (900 to 1800 ms latency as in Figure 1 panel A) with a 100 ms baseline before onset (total epoch duration: 1000 ms). An artifact rejection criterion of ±100 μV was applied offline at all 19 electrodes. Data were re-referenced offline to the common average reference and were 0.18–40 Hz band-pass filtered and 50 Hz notch filtered.

Multivariate decoding of single-trial EEG

We used a single-trial topographic analysis (STTA; Tzovara et al., 2012) to analyze EEG responses to sounds and the EEG activity in the absence of physical stimuli. This method is based on extracting time periods and prototypical voltage topographies discriminating most accurately between two conditions (Tzovara, Murray, et al. 2012). In this specific setting applied to comatose patients, the STTA has the advantage of being free of *a priori* hypotheses about the electrode location where a stimulus-related activity would be expected, and is independent of any inclusion criteria aside from having sufficient artifact-free trials. Moreover, because this method is based on voltage topographies, we can interpret an accurate performance as the result of the activation of different underlying neural generators between experimental conditions (Tzovara, Murray, et al. 2012).

The STTA is based on estimating the voltage topographies of the single-trial EEG by a Mixture of Gaussians model. One part of the dataset is kept aside from the analysis for final validation of the decoding performance (validation dataset). The rest of the dataset is divided in n splits and

used to train the decoding algorithm on *n*-1 data splits and test on the remaining one. The training and testing is repeated *n* times in a way that the decoding is always evaluated on *n* non overlapping test datasets. According to the maximum number of available artifacts-free trials for each condition, the value of *n* was 8 when 80 trials could be used for the training dataset and 7 when fewer trials were available. This procedure is repeated for several parameters values (i.e. number of Gaussians in the mixture) in the model in order to select the values maximizing the decoding performance. The final decoding value is computed on the validation dataset using trials that were never used either for training the model or for the model parameters selection. All values reported below refer to the validation datasets.

The decoding performance is measured as the area under the Receiver Operating Characteristic curve (AUC; Green & Swets, 1966). The significance of the decoding performance is then assessed by comparing its value to the chance level computed by randomly permuting the labels of the single trials used for training the algorithm and recomputing the mixture of Gaussians models 200 times for each recording. The AUC on the validation dataset was compared to the distribution of the AUC values based on these random permutations (Wilcoxon signed-rank test, $p < 0.001$).

Occurrence and specificity of the reactivation effect

Single patient decoding analysis

We performed the EEG analysis in three steps.

1. The first step identified recordings showing evidence of preserved auditory processing (i.e. significant auditory discrimination). We trained the decoding algorithm to distinguish between the EEG activity elicited by the UCS vs. the EEG activity elicited by the neutral silence (Figure

1, panels A and C). The significance was assessed on a separate validation dataset (see previous section). A significant decoding was considered as an indication of preserved auditory processing and provided the statistical models to be used in the second step.

2. The second step aimed at assessing the presence of a reactivation effect, defined as the reactivation of the EEG activity elicited by the UCS (in reinforced trials) at the corresponding silent period in non-reinforced trials ("conditioned silence"). We tested this hypothesis in recordings showing significant auditory discrimination (cf. step 1) by applying the above-mentioned statistical models to discriminate the EEG activity elicited by the conditioned silence (supposed to resemble to the one elicited by the UCS) from the EEG activity during the neutral silence (Figure 1, panels B and C). We considered that the reactivation effect was present when the decoding algorithm discriminated significantly ($p < 0.001$) conditioned silences and neutral silences at the beginning of the experiment (first 20 trials). This criteria was chosen according to previous studies showing that conditioning without consciousness may vanish fast in the time-course of the experiment (Raio et al. 2012).

3. In the third and last step, we assessed the temporal specificity of the reactivation effect, i.e. we checked that reactivation was specific to the precise time-window where the UCS is expected. We considered two silent intervals where no reactivation of the UCS is expected: before UCS onset (during ISI) and after UCS termination (during ITI). To this aim, we extracted epochs of 1000 ms duration (same duration as in previous analyses) spanning from 100 ms to 1100 ms latency for ISI and from 1800 ms to 2800 ms latency for ITI (Figure 1, panels B and C). The same decoding analysis (cf. step 2) was applied to these two intervals.

All analyses explained above (steps 2 and 3) were repeated for the last 20 trials of the experiment to check the persistence of the reactivation effect over time.

Group level statistical analysis

We assessed the temporal specificity of the reactivation effect and its evolution over time in the group of patients showing a reactivation effect by performing a 3 x 2 repeated measure ANOVA with interval (ISI, UCS interval, ITI) and moment of the experiment (beginning vs. end of the experiment) as within-subject factors. Two-tailed paired sample t-tests were applied post-hoc to identify the direction of effects. These tests were run using SPSS Statistics version 22.0 (IBM 2013).

Topographic consistency test (TCT)

An additional topographic consistency analysis was performed in recordings showing a significant reactivation effect (Koenig & Melie-García 2010) to quantify the degree of consistency of a given topography across trials. This test allows to assess statistically the presence of an evoked potential at each time frame based on a randomization analysis. This test generates a non-parametric p-value corrected for multiple comparisons indicating the probability by which a given topography could be generated by chance. We applied it here at UCS interval (800-1800 ms latency) in reinforced, non-reinforced and unpaired trials to investigate whether a consistent evoked response could be identified in the absence of any physical stimulus in non-reinforced trials. We considered the presence of an evoked response when a continuous period of at least 30 ms was significant on the TCT analysis.

Clinical factors in relation to the reactivation effect

To assess whether the occurrence of a reactivation effect is indicative of survival or is influenced by clinical factors, we compared patients showing a reactivation effect vs. the others considering several clinical characteristics. We performed two-tailed independent sample t-tests on continuous variables, including age, time to return of spontaneous circulation (time to ROSC), and temperature at the moment of the recording. We tested categorical variables such as survival at three months, etiology and FOUR score using Fisher's exact test.

RESULTS

Occurrence and specificity of the reactivation effect

Single patient decoding analysis

1. The first step of analysis identified significant auditory discrimination for UCS vs. neutral silence in 14 out of 29 patients, seven of them during TH and nine during NT (16 recordings; two patients showed significant results both during TH and NT). The average decoding performance across all significant results in the validation dataset was 0.67 ± 0.03 while chance level was 0.60 ± 0.02.

2. In the second step, nine recordings (five in TH, two in NT during Day 1 and two in NT during Day 2) from eight patients reached our criteria for a reactivation effect (see Figure 2 for an exemplar patient). The average decoding performance over the nine recordings was 0.64 ± 0.02 (light grey bar at UCS interval in Figure 3) and chance level was 0.5 ± 0.00.

3. The third step of analysis, assessing the occurrence of the reactivation effect before (i.e. during ISI) and after (i.e. during ITI) the interval where the reactivation effect is expected (UCS interval) provided on average lower values, but few recordings showed significant decoding results (Table 1, "ISI" and "ITI" columns).

Concerning the persistence of the reactivation effect over the duration of the experiment, we observed significant decoding results at the end of the experiment in five out of the nine recordings (two in TH; see Table 1 column "UCS interval", sub column "End").

Group level statistical analysis

As displayed in Figure 3, the 3 x 2 ANOVA showed a main effect of interval ($F_{(2, 16)}$ = 8.49, p = 0.003), UCS interval providing the highest decoding values (mean: 0.60 ± 0.02) compared to both ISI (mean: 0.48 ± 0.05; $t_{(8)}$ = 5.51, p = 0.001) and ITI (mean: 0.49 ± 0.02; $t_{(8)}$ = 2.67, p = 0.03). No main effect of moment within the experiment was found, although the decoding performance was lower at the end of the experiment than at the beginning of the experiment for all intervals except for ITI. Exact decoding values for the three intervals (ISI, UCS interval, ITI) and the two moments (beginning / end of the experiment) in patients showing a reactivation effect can be found in Table 1.

TCT results

The topographic consistency test applied at the latency of the UCS revealed that all nine recordings except one showed at least one significant continuous period of 30 ms in reinforced trials, demonstrating a consistent evoked response to the UCS. At the corresponding silence period, six recordings showed an evoked response in non-reinforced trials ("conditioned silence") and four in unpaired trials ("neutral silence"). In addition, two of the patients showing a reliable evoked response in both non-reinforced and unpaired trials exhibited significant results over longer lasting periods in non-reinforced trials in comparison to unpaired trials (see Figure 2 for the TCT results of an exemplar patient). All together these results suggest a more reliable evidence of evoked-like activity in conditioned silences in comparison to neutral silences, consistently with the presence of a reactivation activity.

Clinical factors in relation to the reactivation effect

The level of consciousness of patients showing a reactivation effect was on average 2 ± 0.6 points for hypothermic recordings and 2.8 ± 1.1 for normothermic recordings (FOUR score). All these values indicate a deep coma state (Wijdicks et al. 2005).

As displayed in Table 2, hypothermic patients showing a reactivation effect in hypothermia did not differ from hypothermic patients without reactivation effect on consciousness state or any other clinical variable considered. Moreover, as survival at three months did not differ between the two groups, these results suggest that the occurrence of a reactivation effect is not predictive of outcome. Given the reduced number of patients showing a reactivation effect in normothermia (two on Day 1, two on Day 2), the same tests could not be performed for the subsequent days.

DISCUSSION

Our study aimed at detecting preserved learning capacity in the unconscious human brain. To this aim we recorded EEG in post-anoxic comatose patients during the presentation of an auditory trace conditioning paradigm and we tested the reactivation of the neural activity in response to an alerting sound during the interval where this stimulus is expected but not presented. We found evidence of reactivation in one third of the comatose patients based on significant decoding performance in classifying the spontaneous EEG activity following the CS+ vs. that following the CS-. In these patients, the reactivation phenomenon exhibited a high degree of temporal specificity as shown by a significantly better decoding performance at the latency where UCS is expected than at adjacent time periods. In other words, these patients developed an expectation not only of the occurrence of a specific event, but also of the timing of its occurrence. Consistently, topographic consistency analysis showed longer lasting time periods of evoked responses after the CS+ rather than after the CS-.

In the present cohort, all patients were in acute coma and with a very low score in clinical consciousness scales. Moreover, the majority of the recordings showing a reactivation effect took place during the first day (i.e. in seven recordings out of nine) of coma, five of them under hypothermia and sedation, a state of deep unconsciousness. None of the clinical descriptors could explain the difference between patients exhibiting trace conditioning and the rest (Table 2) and we found no straightforward relation between the occurrence of a reactivation effect and patients' outcome. Together with previous studies showing high decoding results on the first day of coma in auditory discrimination of local and global deviants (Tzovara et al. 2013; Tzovara et al. 2015) as well as in the semantic categorization of sounds (Cossy et al. 2014), these results

suggest that the degree of preserved cognitive functions in this very early stage of coma is not always informative of the severity of the clinical condition.

This could also be explained by a preservation of the neural processes underlying trace conditioning in the very acute stage of coma (i.e. on the first day of coma) which will degenerate over time, a phenomenon that was already evident in other auditory protocols with the same type of patients, and especially in non survivors (Tzovara et al. 2013; Tzovara et al. 2015; see also Piarulli et al 2015). Alternatively, it could be interpreted as the consequence of saturation of the conditioning over subsequent days, as some of these patients were recorded over two or more consecutive days. A decay of the trace conditioning over time is in accordance with previous literature focusing on fear conditioning at different levels of perceptual awareness of the conditioned stimulus (Raio et al. 2012): fear conditioning occurs no matter whether subjects could perceive the stimulus consciously or not, however its persistence over time was crucially dependent on whether the threat was consciously perceived.

Overall, our study showed evidence of a reactivation effect in eight of 29 patients or nine of the 43 recordings performed (21%). Although this number can seem low, it is consistent with results from other studies in the field of disorders of consciousness testing the integrity of various sensory and cognitive functions (Morlet & Fischer 2014; Daltrozzo et al. 2007). In this clinical population, electrophysiological responses to auditory stimuli have been extensively investigated through oddball paradigms allowing to explore the so called mismatch negativity component (MMN) which is tpically identified in less than half of the tested patients (Fischer et al. 2000; Fischer et al. 2004; Kotchoubey et al. 2005). In more complex auditory paradigms, multivariate decoding algorithm showed significant classification performance in response to auditory global

deviants in 14% of vegetative state patients (King et al. 2013) and in 25% of recordings in comatose patients (Tzovara et al. 2015). Evidence of semantic discrimination was also found in acute coma, with 14/38 recordings (37%) showing distinct EEG activity in response to human vs. animal vocalizations and 11/38 (29%) to living and man-made sounds (Cossy et al. 2014). Closer to our study, Signorino et al. (1995) elicited the P300 novelty component in 9/16 patients (56%) in acute coma using patients' own name in a conditioning procedure (Signorino et al. 1995). A reduced number of significant patients is thus common in the field of disorders of consciousness and can be at least partially explained as variations in the severity of patients' medical condition.

Many previous studies investigating the extent of conditioning in unconscious condition were based on subliminal stimulus perception. By varying the degree of perceptual awareness of the CS+ through masking, Balderston et al. (2014) showed that unperceived conditioned stimulus could still elicit a conditioned response (Balderston et al. 2014; see also Esteves et al. 1994 for similar results). Along the same line, a continuous flash suppression paradigm provided further evidence of conditioning with subliminal stimuli (Raio et al. 2012). However the reliability of awareness threshold of subliminal stimuli is limited by the inherent variability of subjects' perception sensitivity upon repeated presentation of the same stimulus, and might explain why other studies reported opposite results arguing for the crucial role of awareness in trace conditioning (Knight et al. 2003; Weike et al. 2007; Asli et al. 2009; see also Pessoa 2005 on the difficulty on an objective assessment of perceptual threshold). Another approach used a concurrent task to diminish the attentional resources and demonstrated a modulation of the degree of learning as a function of attention (Carter et al. 2003; Lovibond & Shanks 2002); even in this case, the difficulty relies on the absence of a general consensus on the assessment of the

dedicated attentional resources and its reliability. Our study relates more directly to other two recent papers showing learning in altered state of consciousness where conditioning was assessed via peripheral measurements (Bekinschtein et al. 2009; Arzi et al. 2012).

Our study adds the extra value of assessing trace conditioning directly at the neural level, avoiding the measure of autonomic or behavioral responses that might be impaired in unconscious and sedated individuals. To the best of our knowledge, previous electrophysiological studies have exclusively investigated trace conditioning in conscious subjects. These previous studies have primarily emphasized an enhanced sensory processing for the CS+ compared to the CS- (Liu et al. 2012; Stolarova et al. 2006; Bröckelmann et al. 2011). This sensory enhancement appeared already at early latencies of stimulus processing irrespective of the sensory modality and implicates the activation of subcortical structures including hippocampus, insula and amygdala (Miskovic & Keil 2012) and even when the conditioning is elicited by masked stimuli (Wong et al. 2004). Even though modulations in the processing of the CS+ indicate that an association has been established, these results do not provide information about the mechanisms allowing the storage and maintenance of the acquired representation after the CS+ processing terminates. In our study we made an explicit hypothesis that this learning phenomenon can be attested by the reactivation of the neural activity belonging to the expected stimulus, allowing also the investigation of the specificity of temporal contingency of the learned association.

Previous literature has provided ample evidence of modulation in neural activity within the sensory areas of the expected stimuli after presentation of a series of stimuli following specific

rules (Kastner et al. 1999; Summerfield et al. 2011; Larsson & Smith 2012; McNally et al. 2011). This evidence has been explained within the general framework of predictive coding. This theory formalizes the role of higher areas of the cortical hierarchy in formulating a prior probability of the incoming sensory stimuli by collecting information over time from sensory cortices. This flow of information is constantly updated over time from lower areas up to higher levels in the hierarchy in order to compare prior expectation with current evidence (Friston 2005). In the particular case of a missing stimulus which was strongly expected based on previous experience, the brain can produce an activity strongly resembling the neural response to the missing stimulus as a manifestation of its internal expectation (Sanmiguel et al. 2013; Chouiter et al. 2015) even when the expectation is built upon unperceived or task-irrelevant stimuli (Den Ouden et al. 2009). Along the same line, predictive coding has been suggested to hold even for unconsciously processed target stimuli (Vetter et al. 2014). Our study complements and extends these findings by showing that the prediction can take place in complete absence of consciousness based on single-patients' internal models of expectation of a relevant arousing stimulus.

Future experiments will investigate the level of generalization of the learned representation. The current evidence leaves unresolved whether patients had established an association between the CS+ and the UCS by keeping a memory trace of the physical features of those stimuli or whether this association was established along other dimensions of the UCS, such as emotional value, alerting features or semantic characteristics (i.e. being an alerting sound). Testing trace conditioning based on a larger variety of sounds would help clarifying the flexibility and the level of generalization that the brain can achieve in implicit learning without conscious perception of the incoming stimuli.

A further unresolved question relies on the dependency of the reactivation effect on having a fixed temporal interval between the CS+ and the UCS. It is possible that consciousness is required to adjust flexibly the probability of receiving salient stimuli over time based on the repeated presentation of the CS+ and UCS at variable temporal intervals.

Alternatively, learning with conditioning in the absence of consciousness might be only possible in a very repetitive and redundant experimental context both in terms of stimuli and temporal gaps. Further investigation of the learning capacity during coma will help defining the boundaries of the learned association in the absence of consciousness and will provide new insights on the preserved cognitive capacities in these patients as a function of time from coma onset.

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LEGENDS

Figure 1: Experimental design. The differential trace conditioning paradigm consisted of three types of trials, each of them starting with a tone (CS+ or CS-). In Reinforced trials (panel A), CS+ is followed by UCS, while in Non-reinforced trials (panel B) and Unpaired trials (panel C), the tone (CS+ and CS- respectively) is followed by a period of silence of the same length as the UCS. ISI stands for Inter-stimulus interval and ITI for Inter-trial interval.

Figure 2: Auditory evoked potentials (AEPs) for reinforced, non reinforced and unpaired trials (A, B and C respectively) in an exemplar patient showing a reactivation effect during hypothermia. Blue dashed lines indicate the CS+ or CS- onset and offset and orange dashed lines indicate the timing of UCS, conditioned silence or neutral silence respectively. 1) AEP across the electrodes montage for the whole trial duration and 2) Global Field Power (GFP) indicating the strength of the electric field; superimposed grey bars show the periods of significance of the TCT analysis.

Figure 3: Average decoding results for the nine recordings showing a reactivation effect. Light grey and dark grey bars indicate the average decoding performance for the comparisons "conditioned silence vs. neutral silence" at the beginning and at the end of the experiment respectively for the three within-trial considered intervals (ISI, UCS interval and ITI). The reactivation effect is displayed with the light grey bar at UCS interval. A repeated measures ANOVA showed a significant effect of interval, with higher decoding values for UCS interval compared to both ISI and ITI.

Table 1. Summary of the decoding results for the three within-trial intervals (ISI, UCS interval and ITI) and the two within-experiment moments (beginning and end of the experiment) in all patients showing a reactivation effect, split according to the time of the recording (TH / NT). Results of the reactivation effect are highlighted in bold. For each group of results, decoding value, chance level and number of patients providing significant results are indicated.

All hypothermic patients (TH) were recorded on Day 1. During normothermia (NT), two patients were recorded on Day 1 and two on Day 2. One patient showed a reactivation effect for both TH and NT recordings.

Table 2. Clinical description of comatose patients separated according to whether they showed a reactivation effect and to the day of recording. Although patients were recorded over several subsequent days, only one patient had significant results on both Day 1 (hypothermia) and Day 2 (normothermia). Temperature and FOUR score were collected at the moment of the EEG recording. Time between cardiac arrest and return to spontaneous circulation is indicated as ROSC. Patients showing a reactivation effect did not differ trivially from the others based on any of the available clinical variables.

8. Table 1

Table 1. Mean decoding results (standard error of the mean) for the recordings showing a reactivation effect, presented separately for hypothermic (TH) and normothermic (NT) patients.

		ISI		**UCS interval**		**ITI**	
		Beginning	End	Beginning	End	Beginning	End
TH (n=5)	Decoding value	0.52 (0.02)	0.45 (0.03)	**0.62 (0.02)**	0.54 (0.04)	0.46 (0.03)	0.53 (0.03)
	Chance level	0.50 (0.01)	0.50 (0.00)	**0.50 (0.00)**	0.49 (0.01)	0.50 (0.00)	0.50 (0.00)
	Significant recordings	3	1	5	2	2	4
NT (n=4)	Decoding value	0.48 (0.06)	0.46 (0.07)	**0.67 (0.04)**	0.58 (0.07)	0.49 (0.05)	0.49 (0.07)
	Chance level	0.51 (0.00)	0.50 (0.01)	**0.51 (0.01)**	0.51 (0.01)	0.50 (0.01)	0.51 (0.01)
	Significant recordings	1	1	4	3	2	2

8. Table 2

Table 2. Comparison of clinical characteristics in patients showing significant reactivation effect or not, split by day and temperature.

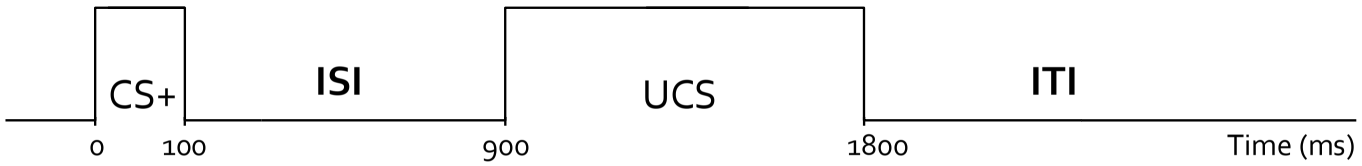
	Patients with reactivation effect	**Patients without reactivation effect**	**t-value (p value)**
Hypothermia (Day 1)	**n = 5**	**n = 14**	
Patient alive at 3 months	80%	71%	(1)
Age (years)	71 ± 5	64 ± 3	1.10 (0.29)
Time to ROSC (min)	26 ± 8	21 ± 4	0.56 (0.58)
Cardiac etiology	100%	85%	(1)
FOUR score ≤ 4	100%	100%	(1)
Temperature (°C)	34 ± 0.2	34 ± 0.2	-1.27 (0.22)
Normothermia (Day 1)	**n = 2**	**n = 5**	
Patient alive at 3 months	50%	20%	
Age (years)	63 ± 8	75 ± 6	
Time to ROSC (min)	4 ± 1	24 ± 9	
Cardiac etiology	100%	80%	
FOUR score ≤ 4	100%	60%	
Temperature (°C)	35 ± 1.8	36 ± 0.2	
Normothermia (Day 2)	**n = 2**	**n = 11**	
Patient alive at 3 months	0%	54%	
Age (years)	77 ± 4	69 ± 3	
Time to ROSC (min)	19 ± 10	23 ± 5	
Cardiac etiology	100%	82%	
FOUR score ≤ 4	50%	64%	
Temperature (°C)	36 ± 0.7	37 ± 0.2	
Normothermia (Day N)[†]	**n = 0**	**n = 3**	
Patient alive at 3 months		25%	
Age (years)		63 ± 8	
Time to ROSC (min)		14 ± 2	
Cardiac etiology		100%	
FOUR score ≤ 4		75%	
Temperature (°C)		37 ± 0.4	

ROSC return of spontaneous circulation; *FOUR* full outline of unresponsiveness (Booth et al., 2004).

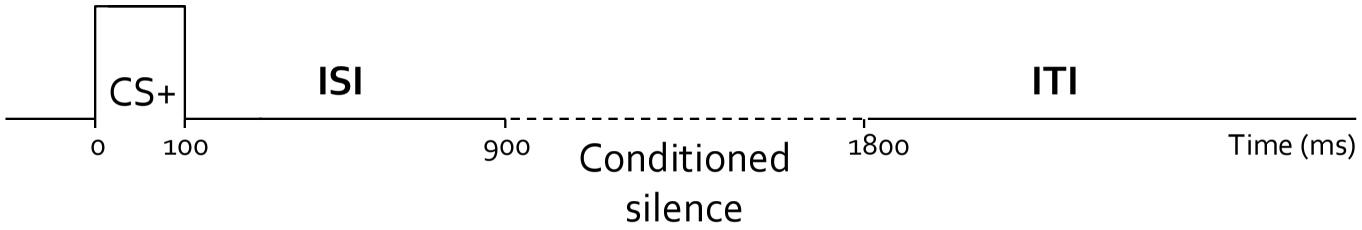
[†] A total of four EEG recordings were performed in three patients. Two recordings took place on Day 3, one on Day 4 and one on Day 6.

9. Figure 1
Click here to download 9. Figure: Figure1_paradigm.pdf

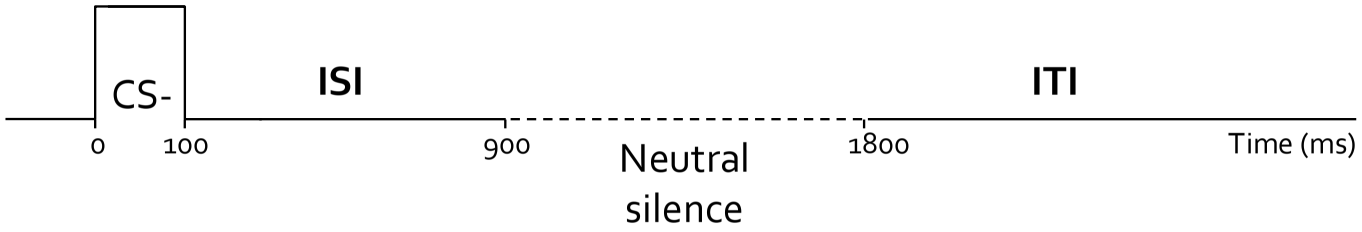
A. Reinforced trials



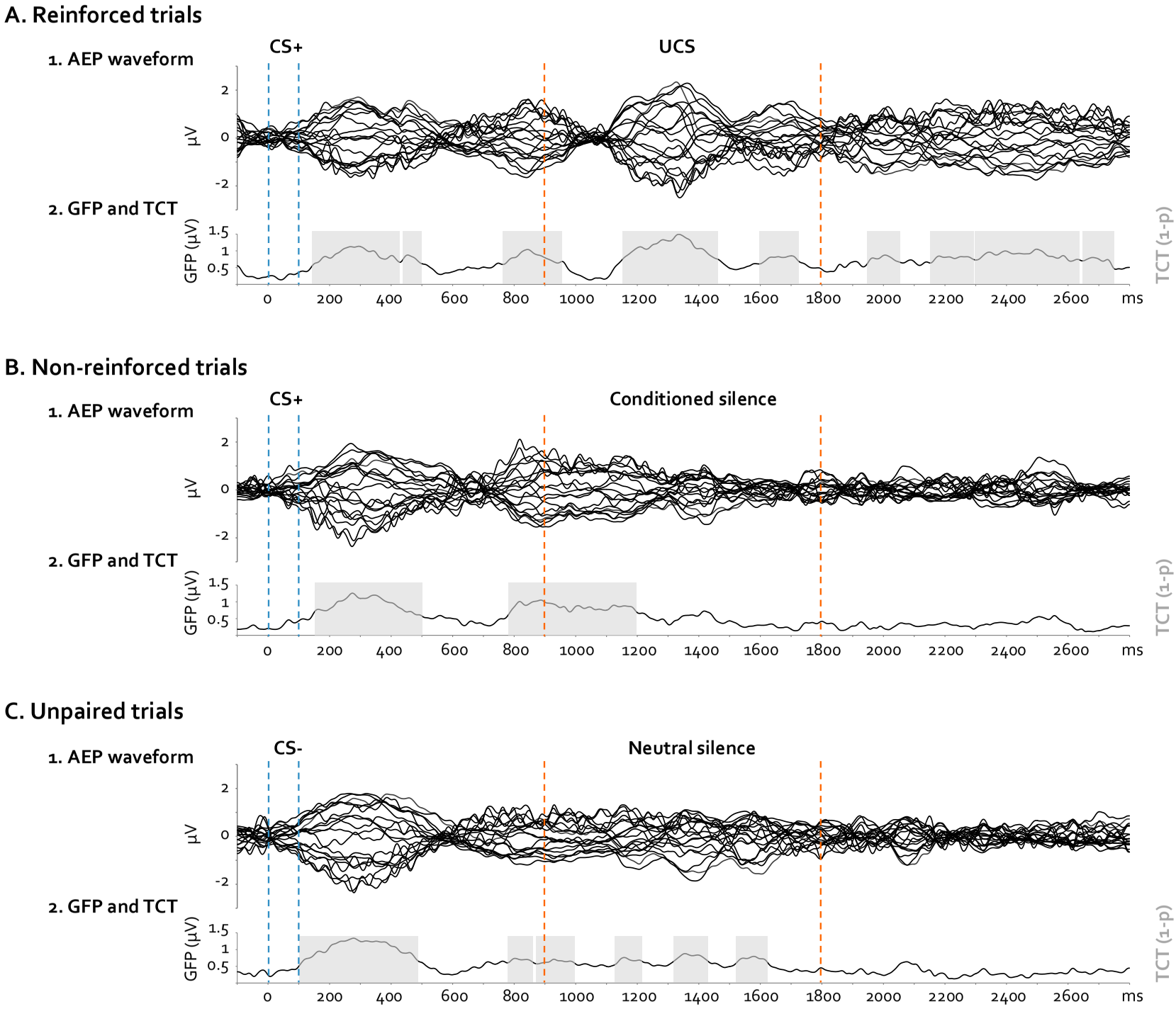
B. Non-reinforced trials



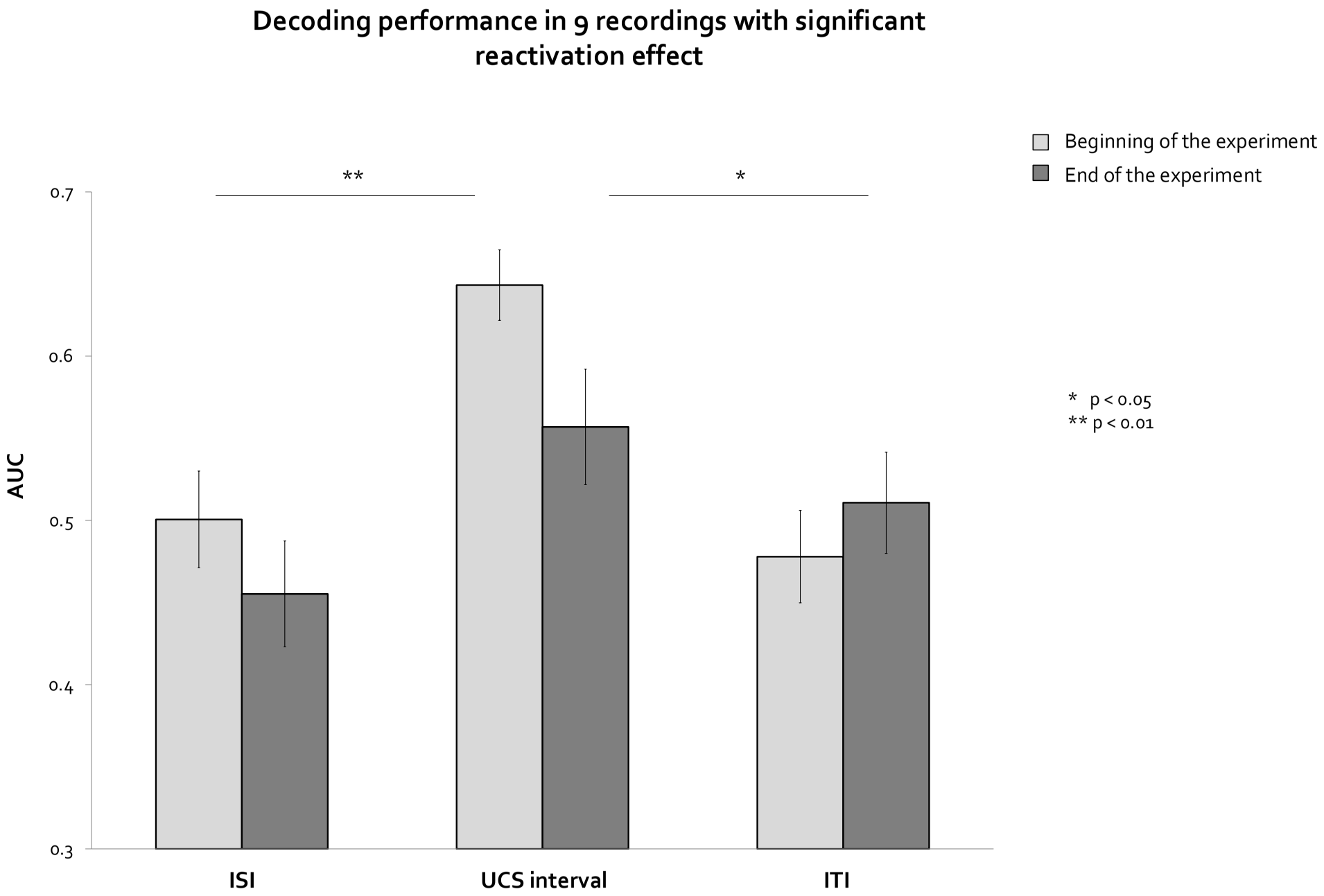
C. Unpaired trials



9. Figure 2
Click here to download 9. Figure: Figure2_ERP.pdf



9. Figure 3
Click here to download 9. Figure: Figure3_group.pdf



SUPPLEMENTARY MATERIAL

Behavioral study conducted to select UCS

We conducted this behavioral experiment for selecting the alerting sounds to be used as UCS in the subsequent electrophysiological experiment.

Twenty healthy subjects (9 women; age: 34 ± 3 years, where here and in the following ± standard error of the mean – SEM – is indicated for all averages) participated to the behavioral experiment. No subjects had history of neurological or psychiatric disease, and all reported normal hearing.

Participants were presented with a set of 34 complex environmental sounds selected from available databases (www.soundjax.com; www.soundbible.com) and from the International Affective Digitized Sounds database (Bradley & Lang, 2007). Half of the sounds consisted of alarms or bells, the other half were randomly selected among animal vocalizations and sounds of objects. All were 16-bit stereo sounds sampled at 44.1 kHz and 900 ms duration. We applied an envelope of 50 ms increase time at the beginning and 50 ms decay time at the end of the sound, using Adobe Audition 2.0. Sounds were further normalized based to the root mean square of their amplitude.

Each sound was presented twice within a randomized sequence. The sequence was displayed via insert earphones (model ER-4P; Etymotic Research) at a comfortable volume level using E-Prime 2.0 software (Psychology Software Tools, Pittsburgh, PA). Participants were asked to rate each sound on three emotional dimensions (valence, arousal and control) using the 9-points non verbal Self-Assessment Manikin scale (SAM) (Lang, 1980; Bradley & Lang, 1994). Although

there was no time limit to respond, participants were encouraged to give their first spontaneous response as quickly as possible. Based on the mean scores of the 40 ratings collected (20 participants x 2 ratings), three sounds were selected for their higher score on arousal dimension (7.2 ± 0.05) compared to the others (5.8 ± 0.2). These three alerting sounds were used as UCS for the EEG based auditory trace conditioning paradigm described in the manuscript.

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